Identification of a Heterozygous Microsatellite Marker in *Pinus pumila*

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Summary *Pinus pumila*, which habits around the timber line in Japanese mountains, propagates from adventitious roots of creeping or belowground stems and from seeds that are dispersed by animals. In an attempt to detect heterozygous simple sequence repeat (SSR) alleles in the nuclear genome of *P. pumila*, which may be useful to identify individuals in natural populations, we performed PCR using the DNA from this species and primer sets that have been reported for *P. strobus* SSR (Echt *et al.* 1996). Aligned subclone sequences originating from RPS150 product suggested that this locus included at least two [ga(t/g)]_n alleles, [gat]₃[gag]₃ and [gat]₃[gag]₁[gat]₁, while the direct sequence of RPS160 product included a single SSR allele of [acag]₃[gcag]₁[acag]₃. This is the first report of the heterozygous nuclear SSR locus in *P. pumila*.

Keywords Microsatellite marker, PCR, *Pinus pumila*, Simple sequence repeat.

Pinus pumila is a member of five-needle pines, which are assigned to subgenus *Strobus*. Natural flora of this species is in the Far East surrounding the Japan Sea (Goncharenko *et al.* 1992). *P. pumila* proliferates sexually (Kajimoto *et al.* 1998) or vegetatively (Kajimoto 1992, Araki 1998, Tani *et al.* 1998). The ratio of these two proliferations in natural populations has been studied by synchronization pattern of annual shoot elongation (Araki 1998) or by allozymes, random amplified polymorphic DNA and inter-simple sequence repeat (Tani *et al.* 1998). However, the dominance of either propagation in populations has been difficult to determine. Nuclear SSR markers are transmitted into progenies according to the Mendelian law. Then tracing SSR in a population can detect the occurrence of mating in populations (Beaulieu and Simon 1994, Tani *et al.* 1996, Iwasaki *et al.* 2013). With the development of nucleotide sequencing technology, identification of SSRs has become within the reach of plant ecologists. Nuclear SSRs consisting of two-, three- or four-base units were reported in a *Strobus* species, *P. strobus* (Echt *et al.* 1996, 1997). In the annealing of PCR amplification, three- or

four-base unit of SSRs may generate less slippages resulting in less nucleotide-sequence errors in DNA synthesis than two-base unit of SSRs.

In order to survey heterozygous SSR loci in *P. pumila*, we performed PCR using genome DNA in this species and primers that are reported to amplify three- or four-base units of SSRs in *P. strobus*. Based on the results obtained, we discuss the possible application of these SSR markers in future studies.

Materials and methods

Sample collection

Leaves of *P. pumila* were collected in Autumn 2018 from a shrub located at latitude 36.114373° North and longitude 137.549717° East, which is 2776 m above sea level on Mt. Norikuradake. In a point at latitude 36.122462° North and longitude 137.555346° East, where is 443 m North-northeast of the above-mentioned site, another shrub was photographed December 18, 2019.

DNA extraction, PCR amplification, and nucleotide sequencing

The genome DNA was extracted according to a previous method (Uchida *et al.* 2007) with a slight modifi-

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cation from original protocol (Murray and Thompson 1980). Using 0.17 g in fresh weight of leaves, which had been stored in -80°C , $0.22\mu\text{g}$ of the genome DNA was extracted. This extraction yield was at the same level in another extraction. PCR was performed using genome DNA, primer sets of either RPS150 or PRS160 (Echt *et al.* 1996) and Ex Taq Hot Start Version (TaKaRa). PCR cycle for RPS150 amplification was as follows; one cycle of 94°C for two min, 40 or 45 cycles of 95°C for 30 s, 52°C for 30 s and 72°C for one min and one cycle of 72°C for 10 min. That of RPS160 was the same as in RPS150, except the annealing temperature of 55°C . After removing primers in amplified products using UltraClean PCR Clean-Up Kit (MO BIO), direct sequencing was performed using either forward or reverse primers that were used for PCR unless otherwise stated. In an attempt to obtain a better forward RPS150 direct sequence, a 0.25-kb PCR product was excised from the gel, purified and was subjected to direct sequencing again. To obtain individual sequences in the PCR product of RPS150, this DNA fragment was cloned into pBluescript II KS (+) using NEBuilder HiFi DNA Assembly Master Mix (NEB). Forward and reverse PCR primers for the amplification of the overlapping insert are 5'-tagggcggaattgtccatcagtgagcagtggt-3' and 5'-gaacaaaagctgcacttgggtcttcttccc-3', respectively (sequences annealing to insert are underlined). Those of the overlapping vector is 5'-gaagcccaagtgcagcttttgtcccttagt-3' and 5'-ctcactgatggacaattcgcctatagtgagt-3', respectively (sequences annealing to vector are underlined). In order to screen positive *Escherichia coli*

clones, colonies obtained were subject to PCR amplification using vector-specific forward and reverse primers of 5'-gatgtgctgcaaggcgattaag-3' and 5'-agttagctcactcattaggcac-3', respectively, followed by another PCR using insert-specific forward and reverse primers of 5'-tccatcagtgagcagtggt-3' and 5'-cacttgggtcttcttccc-3', respectively. Plasmids were isolated from positive clones using GenElute Plasmid Miniprep Kit (Sigma). The independent clones were subjected to dideoxy sequencing. Sequences obtained were aligned using MAFFT version 7 online (<https://mafft.cbrc.jp/alignment/software/>).

Results and discussion

Figure 1 shows the shrub of curved and dwarf stems of *P. pumila* in Mt. Norikuradake (Fig. 1A). From a stem of this bush, leaves were sampled for DNA extraction. On the ground, tiny seedlings were formed adjacent to distributed cones (Fig. 1B). In another place, 443 m away from this spot, densely developed creeping stems of *P. pumila* were observed (Fig. 1C).

Figure 2A shows PCR amplification of RPS160 locus in *P. pumila* genome. In the PCR reaction with forward and reverse primers added, a 0.25-kb fragment was detected, while no specific bands were formed in the negative controls of no primer addition or of only one primer addition. In most region of electropherograms in forward and reverse direct sequences, Phred-quality scores were in higher levels (data not shown). Figure 2B shows parts of forward direct sequence in RPS160

Fig. 1. Dense bush of dwarf trees of *P. pumila* is observed on Mt. Norikuradake (A). At the base of this bush, cones (arrowheads) and seedlings (arrows) are observed as well as mosses (B). Densely developed creeping stems (arrows) were exposed on the slope of this mountain (C).

Fig. 2. PCR amplification of RPS160 and RPS150 loci and corresponding nucleotide sequences from *P. pumila* genome. The amplified RPS160 product (A), direct RPS160-forward (B) and RPS160-reverse (C) sequences, amplified RPS150 product (D), direct RPS150-forward sequence (E) and alignment of 12 RPS150-forward subcloned sequences (F). In A and D, lanes a, b, c and d show products with no primers added, with only forward primer added, with only reverse primer added and with forward and reverse primers added, respectively. Black bars with numbers indicate positions and sizes of DNA size markers. In B, C and E, arrays of blue vertical lines above sequences show the highest levels of Phred-quality scores, while yellow and red bars, such as in the right half of E, exhibit lower and lowest levels of scores, respectively. Lower levels of Phred-quality scores in the upstream of C, are due to initiation of sequencing. Brown bars above nucleotide sequences within parentheses show positions of simple sequence repeats. In the middle of E, the uppermost and the lowest levels of nucleotide sequences without parentheses are two possible sequences suggested in the following panel and the middle level of nucleotide sequences without parentheses shows coincidence of these two sequences. In F, 12 sequences are categorized into two groups possessing specific sequences that are emphasized with shades with purple or green. The former and the latter groups are referred to as $[ga(t/g)]_6$ and $[ga(t/g)]_5$ alleles, respectively. Upper and lower boxes along with nucleotide sequences with parentheses suggest predicted simple sequence repeats of $[gat]_3[gag]_3$ and $[gat]_3[gag]_1[gat]_1$, respectively. Nucleotides shaded with gray show conserved ones in more than nine clones.

product, including a simple sequence repeat (SSR) of $[acag]_3[gag]_1[acag]_3$. The corresponding complementary sequence was obtained in high Phred-quality scores when reverse RPS160 primer was used (Fig. 2C). Since species in the genus *Pinus* have a common chromosome number of $2n=24$ (Shibata *et al.* 2016), RPS160 SSR obtained in this study suggests that RPS160 locus consists of the two homozygous SSR alleles, as was reported in counterpart in *P. strobus* (Echt *et al.* 1996, 1997, GenBank accession number U60257.1).

Figure 2D shows PCR amplification when RPS150 primer set was used. A 0.25-kb fragment was amplified when forward and reverse primers were used, while the corresponding band was not detected when no primer or only one primer was added. Figure 2E indicates a part of

an electropherogram showing forward direct sequence derived from PCR reaction mixture from which primers were removed. Phred-quality scores were higher in the upstream of $[gat]_3[gag]_1[ga(t/g)]$ and drastically fell down downstream of this repeat (Fig. 2E). Clear, discrete and higher peaks of nucleotides were observed upstream of this SSR, while different colors of shorter peaks overlapped in the following. This result was reproduced in the direct sequence which derived from a 0.25-kb band excised from the gel. Then we cloned this PCR product into pBluescript KS II(+), performed nucleotide sequencing of 12 independent plasmids and aligned these sequences (Fig. 2F). Two boxes in Fig. 2F suggest two types of $[ga(t/g)]_n$ SSR alleles in RPS150 locus, $[gat]_3[gag]_3$ and $[gat]_3[gag]_1[gat]_1$. This result is in con-

trast to the previous report that *P. strobus* RPS150 locus possesses a single allele having SSR of [gag]₄ (Echt *et al.* 1996, GenBank accession number U60256.1). Though there was a study that detected single-strand conformation polymorphisms in *P. pumila* nuclear genome (Watano *et al.* 2004), there have been no reports to detect different sequences of an SSR allele. This is the first report showing heterozygous SSR sequence in this plant.

Characterization of hetero- or homozygous loci in seeds or in the different generations of seedlings is expected to reveal the dominance of sexual or vegetative proliferation in populations. Future analyses of RPS150 locus in more samples from the field may cast insight to reveal this problem (Kajimoto 1992, Tani *et al.* 1996, Araki 1998). Since intra-species crossing is often observed in *Pinus* species (Watano *et al.* 1996, Senjo *et al.* 1999, Gugerli *et al.* 2001, Goroshkevich 2004, Watano *et al.* 2004, Ito *et al.* 2008, Iwasaki *et al.* 2013), RPS150 locus can also be traced in the hybrids from *P. pumila* and its relatives to evaluate industrially important lines such as in pest resistance, lumber production, edible seed supply or bonsai breeding.

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