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Isolation and characterization of novel microsatellites for *Abies koreana* and *A. nephrolepis* (Pinaceae)

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ABSTRACT. *Abies koreana* is an endemic and rare species from Korea and is classified as endangered by the International Union for Conservation of Nature. Although the genetic diversity assessment for current population of *A. koreana* needs to be performed urgently, no microsatellite markers have been developed for this species. In the present study, we developed 22 novel polymorphic microsatellite loci and the characteristics of these loci were determined in *A. koreana* as well as in *Abies nephrolepis*, the most closely related species, and these loci were compared with previously reported microsatellite markers developed for the *Abies* genus. Genomic sequence (161 Mbp; 325,776 reads) was obtained from one individual of *A. koreana* using Roche 454 GS-FLX Titanium sequencing and 19,258 repeat motifs were identified from it. A total of 288 primer pairs with high copy numbers of di-repeat motifs were evaluated for amplification in *A. koreana* and *A. nephrolepis*. A total of 71 primer pairs successfully amplified fragments, of which 22 showed polymorphisms in *A. koreana* and *A. nephrolepis*. The average expected diversity was 0.767 and 0.717 in *A. koreana* and *A. nephrolepis*, respectively; these heterozygosity levels were

moderate compared to the previously reported microsatellite loci from *Abies* species. This is the first set of microsatellite markers developed for *A. koreana* as well as *A. nephrolepis* and further population genetic studies of both species and genetic delimitation can be carried out for the species conservation and management.

Key words: *Abies koreana*; *Abies nephrolepis*; Microsatellites; Pinaceae

INTRODUCTION

Abies koreana E. H. Wilson, known as Korean fir, is an endemic and rare species from Korea whose distribution is limited to high elevations on the mountains of Jejudo Island and the southern part of the Korean peninsula (Chang et al., 1997; Kim et al., 2011; Hong et al., 2011). This Korean endemic species is classified as endangered by IUCN due to its restricted distribution in a few scattered areas and continuous decrease in natural populations (Kim et al., 2011). Moreover, sudden dried death of this species in Mt. Hallasan, Jejudo Island, was observed from 2012 and this phenomenon is severely reducing its population size in Mt. Hallasan, where the biggest population of *A. koreana* occurs. As an alpine conifer, *A. koreana* shows low photosynthetic rates in higher air temperature in the natural population in Mt. Hallasan (Woo et al., 2008b) and recent reduction in the population of *A. koreana* was caused by the dieback (Woo, 2009), which could likely be the result of changes in multiple environmental factors due to global warming. Since vulnerability of this species to climate changes is considered to be relatively low (Woo, 2009; Hong et al., 2011), *A. koreana* was designated as an indicator species for climate change by the Korean government (Lee et al., 2010). Thus, the genetic diversity assessment for the current population of *A. koreana* needs to be performed soon for its conservation and taxonomic distinction from *A. nephrolepis*.

Abies nephrolepis (Trautv. ex Maxim.) Maxim, known as Manchurian fir, is closely related to *A. koreana* and both the species belong to section Balsamea (Xiang et al., 2015). In addition, these two species are geographically parapatric. *A. nephrolepis* occurs in the central and northern parts of Korea, Manchuria, and Siberia. Although these two species are distinguished by leaf length, bark roughness, and the direction of bract-scales (i.e., downward or otherwise; Wilson, 1920; Chang et al., 1997), none of these characteristics are clearly distinct between the species (Chang et al., 1997; Eo and Hyun, 2013). Several molecular studies have explored the genetic diversity of *A. nephrolepis* (Woo et al., 2008a) as well as the genetic relationship between *A. koreana* and *A. nephrolepis* (Kormutak et al., 2007; Hong et al., 2011). However, population structure and the level of gene flow between the two species were not discussed clearly because of the low abundance of polymorphisms within single chloroplast regions (Kormutak et al., 2007) and the small number of microsatellite markers with a low number of alleles (N_A) (Hong et al., 2011).

Despite the strong demand for identification of co-dominant species, specific microsatellite markers from *A. koreana* have not been identified. To generate microsatellite primers for *A. koreana*, we used next-generation sequencing (NGS) with genomic DNA of *A. koreana* and then used a bioinformatics approach to obtain fragments encompassing repeat motifs from low-depth genomic sequences (Yu et al., 2011; Wang et al., 2014). To use the microsatellite markers for further comparison between *A. koreana* and *A. nephrolepis*, amplification of sequences in both the species and the evaluation of polymorphisms were

performed. In all, 22 new polymorphic microsatellites were identified for both the species. These new markers could be useful for population genetic studies and conservation strategies for *A. koreana* and *A. nephrolepis*.

MATERIAL AND METHODS

Sample collection and DNA extraction

Healthy fresh leaves from 30 *A. koreana* and *A. nephrolepis* individuals, each, were sampled from Mt. Hallasan in the Jejudo Island and Mt. Seoraksan in Kangwon province of Korea, respectively, and dried on silica gel. Dried leaves were ground in a Tissue Lyser II (Qiagen, Hilden, Germany) and total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen). DNA was stored at -20°C until it was used.

454 GS-FLX titanium sequencing and mining repeat motifs

To obtain low-depth genomic sequences, DNA from an individual sampled from Mt. Deokyusan, Jeollabuk Province, Korea was used to generate a genomic library, and the voucher was deposited in the herbarium (KE) of the National Institute of Biological Resources (Incheon, Korea). The NGS library was generated from approximately 10 µg genomic DNA and sequenced on a Roche 454 GS-FLX Titanium platform (454 Life Sciences, Branford, CT, USA) at the National Instrumentation Center for Environmental Management of Seoul National University (NICEM). The obtained sequence reads were assembled using Newbler 2.6 (Roche Diagnostics, Mannheim, Germany) with a 96% minimum overlap identity.

The dinucleotide and trinucleotide repeats of more than four iterations were searched using the Perl program "SSR_finder.pl" (Tóth et al., 2000; Yu et al., 2011). The repeats were sorted according to the number of iterations, and primer pairs flanking each repeat were designed to amplify fragments containing repeats of more than four iterations using Primer 3 (Untergrasser et al., 2012). The optimal primer size was set to a range of 18-26 bases and the optimal melting temperature was set to 58°C. The optimal product size was set to 130-400 bp and the remaining parameters were kept at default settings.

PCR and genotyping

Two hundred eighty eight designed primers were evaluated for amplification by polymerase chain reaction (PCR). PCR was performed in a total volume of 20 µL containing 10 ng DNA from one individual each of *A. koreana* and *A. nephrolepis* and 0.2 µM of each forward and reverse primer using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City CA, USA). The PCR program consisted of the following steps: pre-denaturation (10 min at 95°C), followed by 35 cycles of denaturation (30 s at 95°C), annealing (30 s at 58°C), and extension (30 s at 72°C), and a final extension of 7 min at 72°C using a Mastercycler (Eppendorf, Hamburg, Germany). The amplified fragments and their sizes were visualized using QIAxcel (Qiagen) with the QIAxcel DNA Screening Kit (Qiagen). Sanger sequencing reactions were performed using PCR primers and the ABI PRISM BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) and were sequenced on an ABI 3730XL Analyzer (Applied Biosystems) by Macrogen (Seoul, Korea). Using Sequencer 4.8 (Gene

Codes, Ann Arbor, MI, USA), the integrity and specificity of the PCRs were examined to confirm amplification of the target fragments. Samples in which the alleles were uncertain were verified by repeating the amplification and proofreading the raw data. The primer pairs were used to confirm the targeted repeat sequences, which were further assessed by genotyping all individuals using 5' fluorescence-labeled forward primers (Table 1). Two to four different primers were multiplexed depending on their size differences and the colors of the fluorescent labels. Polymerase chain reactions were performed using the Qiagen Multiplex PCR Kit (Qiagen). The obtained fragments were run on an ABI 3730 DNA analyzer using the Genescan™ 500 LIZ size standard (Applied Biosystems) and analyzed using GeneMarker 1.85 (Gene Codes, Ann Arbor, MI, USA). The primer sets that generated numerous signal peaks or the ones that amplified monomorphic microsatellite loci were excluded.

Table 1. Characteristics of 22 newly developed microsatellite loci in *Abies koreana* and *Abies nephrolepis.*

Locus name	Primer sequences (5' to 3')	Repeat motif	Size range (bp)	Fluorescence dye	GenBank accession No.
AK3	F: CGAAGGAAAGGATGTCATGTAT	$(AG)_{20}$	175-217	6FAM	KT289898
	R: TTGCTCCTCTTTCACTCTTCTC				
AK5	F: AGCTAAAATGCTTTTGGGTGTA	$(AG)_{19}$	291-331	VIC	KP869866
	R: TCTATCAAGCACTGAGAGGGAT				
AK87	F: GCAGCCTTATCTTCATTTTGTC	$(TG)_{15}$	263-294	NED	KT289899
	R: CACTTGAGCCACACTTGAACTA				
AK171	F: GGCATTTGAACACTTACACTGA	$(TG)_{14}$	214-235	PET	KP869867
	R: AGATTTTTGTTGGAATCTGCAC				
AK173	F: GAGACTAGCATATACACCATCGG	$(CA)_{14}$	163-197	6FAM	KT289900
	R: AAGGGAATACACTCAGTCGAGA				
AK174	F: TGATATTTGTGAAATTGGGGTT	$(TG)_{14}$	248-279	NED	KP869868
	R: GTTTCTAAAGTGCAAAATTGGG				
AK176	F: TTACACCGTTAAAAAGGGAATG	$(TG)_{14}$	319-336	VIC	KP869869
	R: CTCATGATGTGTAGCCATTTGT				
AK182	F: TATGATGTGAAACCAATGGCTA	$(TG)_{13}$	367-379	PET	KP869870
	R: AGCTTGGAGTCAAGTTGTCAAG				
AK186	F: TCATTAAGCCACAAATATGACG	$(AG)_{12}$	166-179	6FAM	KT289901
	R: ATCCGTTGTAAGTTCCTCTTCA				
AK238	F: AATTAGGAAAAGGGCGAAGA	$(CA)_{12}$	264-275	PET	KP869871
	R: GCAAAGAAAAACAAACAAAACC				
AK240	F: AGAGAAGGGTCGAGGAATTATC	$(CA)_{12}$	177-212	6FAM	KP869872
	R: TGAAAGTAGCAAGTGTAACTTATGC				
AK246	F: TAGATTGGCATATTGGACATCA	$(TG)_{11}$	135-155	NED	KP869873
	R: ATAGGTTGTTGAGCTGGATGTT				
AK247	F: GGATGGTGCTTTGTTGATATTT	$(TG)_{11}$	166-183	VIC	KP869874
	R: AAATGGTTTGAGCAACATTCTT				
AK252	F: TGCATGTTGTTAGTTGGTAAGG	$(TG)_{11}$	286-306	6FAM	KP869875
	R: TCTAGGTGGAGCAACAAGAGAT				
AK258	F: CATTGGTTGTTCAGGTTATGG	$(CA)_{10}$	212-229	NED	KT289902
	R: GTGGTGCTACCAATGACACA				
AK259	F: CGGTTAAGGGTTTCTAGGGTAA	$(TG)_{10}$	259-280	VIC	KT289903
	R: CCTACTAAAACCCACCCCTATC				
AK263	F: ATTGGATTTAGCTTTGTGGCTA	$(CA)_{9}$	202-206	NED	KT289904
	R: TAGTCTCTCTTCCGTTTGGTGT				
AK264	F: TGTATTGGTAGTCATGCGTTGT	$(CA)_{9}$	214-219	VIC	KT289905
	R: CTAAACTGTGGGGTCAGAAGAG				
AK270	F: CGTGGGGTTTCTAGGGTATC	$(TG)_{9}$	295-302	NED	KT289906
	R: AAGACAACACGCAAAAGTACAA				
AK273	F: TCGGTAGTGGTGAAACGTTATT	$(CA)_{9}$	312-318	VIC	KT289907
	R: AATCAAGTGGCTGCAAGACTAT				
AK284	F: AGAGACCATGGCAAGTTTGA	$(AG)_{10}$	219-225	NED	KT289908
	R: CTACCCAAAACCCAAACAAA				
AK285	F: TATTGTCGATTTGTATGAGGCA	$(AG)_{10}$	206-249	VIC	KT289909
	R: CATAGATCCATCTTAACCCCAA				

Comparison with previously reported microsatellite markers from related species and data analysis

Eight microsatellites developed from *A. sachalinensis* and 13 from *A. alba* were

genotyped in the same populations of *A. koreana* and *A. nephrolepis* (Cremer et al., 2006; Lian et al., 2007). The total N_A, observed heterozygosity (H_O), expected heterozygosity (H_E), both within populations, and the departure from Hardy-Weinberg equilibrium (HWE) were calculated using Arlequin ver. 3.5 (Excoffier and Lischer, 2010).

RESULTS

NGS generated 161 Mb sequence reads (325,776 reads) for *A. koreana*. The total number of contigs was 2,599 and the number of singletons was 282,150. A total of 19,258 regions were found to contain di- or trinucleotide perfect repeats for more than four iterations (dinucleotide repeats: 14,916; trinucleotide repeats: 4,342). AT (42.1%) and ATG (9.5%) repeat motifs were the most common among the di- and trinucleotide repeats, respectively. AT repeat motif is known to occur most frequently among dinucleotide repeats in plant species (Morgante and Olivieri, 1993). The longest repeat motifs were 50 iterations of CA and 20 iterations of ATC.

A total of 288 primers with high copy numbers of all the four di-repeat motifs for amplification and assessment of polymorphisms were synthesized to test the amplification efficiency and to assess polymorphism. Of these, a total of 71 primer pairs (25%) successfully amplified unambiguous alleles after PCR and 22 loci were polymorphic with 2-19 alleles in each species (Table 2). Nucleotide sequences of the 22 novel microsatellite markers were deposited in GenBank under accession numbers KP869866-869875 and KT289898-289909 (Table 1).

Table 2. Characterization of 22 newly developed polymorphic microsatellite loci and those previously reported from the populations of *Abies koreana* and *Abies nephrolepis*.

Locus	*Abies koreana* (N = 30)			*Abies nephrolepis* (N = 30)		
	N_A	H_O	H_E	N_A	H_O	H_E
AK3	15	0.233	0.909*	19	0.467	0.949*
AK5	16	0.800	0.897	18	0.900	0.937
AK87	11	0.467	0.886*	14	0.670	0.920*
AK171	7	0.167	0.570*	6	0.433	0.528
AK173	7	0.433	0.697*	12	0.670	0.886
AK174	11	0.800	0.867	19	0.700	0.910
AK176	7	0.700	0.801	5	0.633	0.737
AK182	4	0.233	0.642*	5	0.467	0.586
AK186	3	0.167	0.158*	7	0.567	0.750
AK238	4	0.533	0.681	6	0.367	0.786*
AK240	6	0.467	0.625	11	0.500	0.677*
AK246	6	0.767	0.737	7	0.533	0.719
AK247	8	0.667	0.780	9	0.867	0.849
AK252	5	0.667	0.744	6	0.200	0.697*
AK258	7	0.267	0.733*	12	0.233	0.896*
AK259	3	0.400	0.341*	3	0.133	0.514*
AK263	2	0.00	0.127*	3	0.267	0.267
AK264	4	0.367	0.567*	2	0.233	0.210*
AK270	5	0.333	0.641*	4	0.367	0.456*
AK273	4	0.067	0.189*	4	0.100	0.245*
AK284	5	0.067	0.753*	3	0.667	0.188*
AK285	16	0.233	0.919*	22	0.633	0.947*
Mean	7.1	0.402	0.767	9.0	0.482	0.717
Microsatellite loci developed previously from *A. sachalienensis*						
As08	6	0.400	0.676*	5	0.367	0.617*
As13	10	0.833	0.724	12	0.767	0.857
As20	7	0.633	0.762	12	0.467	0.858*
As21	12	0.533	0.850*	15	0.567	0.879*
Mean	8.8	0.600	0.743	11.0	0.542	0.857
Microsatellite loci developed previously from *A. alba*						
SF50	6	0.533	0.681	8	0.167	0.637*
SF83	2	0.467	0.506	4	0.467	0.427
Mean	4.0	0.500	0.594	6.0	0.317	0.427

N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity. *P < 0.01 = significance of deviation from Hardy-Weinberg equilibrium after 1000 permutations.

Numbers of alleles, observed heterozygosities and expected heterozygosities for each locus in the populations of *A. koreana* and *A. nephrolepis* shown in the table 2. Similar levels of H_E (H_E = 0.767 for *A. koreana* and H_E = 0.717 for *A. nephrolepis*) were observed in both the species. A total of 14 and 12 loci showed significant deviation from HWE ($P < 0.01$) in *A. koreana* and *A. nephrolepis,* respectively. These deviations were most likely due to excessive homozygotes and the structure within the samples. The total H_O was lower than H_E, with the exceptions of AK186 and AK252 in *A. koreana* and AK247, AK264 and AK284 in *A. nephrolepis*.

DISCUSSION

The polymorphisms in the sequences of the commonly used molecular markers were so low among *A. koreana* and *A. nephrolepis* that the detailed relationships between these two species and further population genetic characteristics cannot be investigated with previous markers (Kormutak et al., 2007; Xiang et al., 2015). In the present study, the high level of polymorphism and heterozygosity of 22 microsatellite loci would be a valuable to investigate further population studies with more extensive samplings from *A. koreana* and / or *A. nephrolepis*. The values of H_E in *A. koreana* and *A. nephrolepis* were moderate in the *Abies* genus (Saito et al., 2005; Cremer et al., 2006; Josserand et al., 2006; Lian et al., 2007; Rasmussen et al., 2008; Sánchez-Robles et al., 2012; Zhan et al., 2014).

While *A. sachalinensis* belongs to same section, *Balsamea*, as do *A. koreana* and *A. nephrolepis* (Farjon and Rushforth, 1989; Xiang et al., 2015), *A. alba* population is relatively distinct from the rest of species and belongs to the section *Abies* (Farjon and Rushforth, 1989; Xiang et al., 2015). In general, cross species microsatellite shows low level of transferability in plants to very closely related species (Primmer et al., 1996; Peakall et al., 1998; Scribner and Pearce, 2000) and reduced levels of diversity in the related target species (Morin et al., 1998; Peakall et al., 1998). The microsatellite loci from *A. sachalinensis* showed reduced amplification success rate (50%, 4 out of 8 loci) and lowered N_A and heterozygosity values in *A. koreana* and *A. nephrolepis* than their values from *A. sachalinensis* (Lian et al., 2007). While the microsatellite loci transferred from *A. alba* revealed very low level of amplification in *A. koreana* and *A. nephrolepis* (15%, 2 out of 13 loci), N_A and heterozygosity values were increased.

We developed 22 new polymorphic microsatellite markers for *A. koreana* and *A. nephrolepis* from the low depth genomic sequences obtained using 454 GS-FLX NGS. This is the first set of microsatellite markers developed for *A. koreana* as well as for *A. nephrolepis* and therefore, with these 22 polymorphic microsatellite markers, further population genetic studies of both the species and genetic delimitation can be carried out for the species' conservation and management.

Conflicts of interest

The authors declare no conflict of interest.

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