

Article

Identification and Development of a Species-Specific Molecular Marker for Discriminating Between *Abies koreana* and *Abies nephrolepis*

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Abstract

Abies koreana Wilson (Korean fir) and *Abies nephrolepis* (Trautv. Ex Maxim.) Maxim. (Khingan fir) are ecologically and economically significant coniferous species in East Asia. However, morphological similarities and hybridization complicate species identification, affecting conservation, forestry management, and commercial activities. Here, we developed a species-specific DNA marker using single nucleotide polymorphisms (SNPs) within the mitochondrial *nad5* intron 1 region to discriminate between *A. koreana* and *A. nephrolepis*. In particular, PCR amplification and sequencing analyses of candidate drought and heat stress-responsive genes as well as mitochondrial *nad5* intron 1 and *nad5* intron 4 revealed a species-specific T-to-G substitution in *nad5* intron 1. Allele-specific primers were designed, and competitive allele-specific labeled light emission technology was employed for SNP detection. The primers were validated using real-time PCR, achieving high specificity and reliability. Overall, the molecular diagnostic tool offers a practical solution for accurate species identification, aiding conservation efforts and sustainable resource management.

Keywords: *Abies koreana*; *Abies nephrolepis*; conservation genetics; *nad5*; species identification

1. Introduction

Industrialization has led to continuous and accelerated global warming through increased carbon dioxide emissions [1]. Various abnormal climate phenomena associated with global warming, such as heatwaves, droughts, rising sea levels, warmer winter temperatures, and the increased frequency and intensity of typhoons have led to extensive habitat loss [2]. Species inhabiting ecosystems that are particularly sensitive to warming, such as alpine regions, are at risk of habitat reduction and population declines, potentially leading to extinction [2]. Given the predicted significant decline in biodiversity due to climate change, there is a growing need for diverse research aimed at managing vulnerable species and ecosystems.

Abies koreana (Korean fir) and other *Abies* species inhabit vulnerable alpine regions [3]. *A. koreana* was first reported as an endemic species of Korea in 1920 and is distributed in high-altitude regions above 1000 m in southern areas, including Mts. Halla, Jiri, Deogyu, and Gaya [4,5]. However, the species rarely forms forests. It is typically limited to small areas or exists as scattered individual trees, except on Mt. Halla, where it makes up wide and pure forests, representing the only large-scale *A. koreana* forest in the world [5,6]. *A. koreana* is classified as endangered [EN B2ab(ii,iii,v)] by the International



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Union for Conservation of Nature (IUCN) owing to its restricted distribution and vulnerability to climate change [7,8]. The species plays a crucial role in maintaining subalpine ecosystem stability, particularly on Jeju Island, where it forms unique forest communities at elevations between 1000 and 1950 m. *Abies nephrolepis* (Trautv. Ex Maxim.) Maxim. (Khang fir) is more widely distributed across northeastern Asia and contributes significantly to forest ecosystem services and timber production in temperate regions [9,10]. Both species are valuable sources of bioactive compounds and essential oils, with applications in pharmaceutical and cosmetic industries [11,12]. However, rapid dieback has been observed due to environmental stresses, including biotic and abiotic stresses [13,14]. Therefore, conservation efforts are essential to protect vulnerable ecosystems in subalpine regions where the species are found.

However, the morphological similarities and potential hybridization between *Abies* species pose challenges for accurate identification, thereby hindering conservation efforts, forestry management, and commercial applications, particularly in seed production and seedling certification programs. Traditional identification methods based on subtle morphological traits, such as cone characteristics, needle arrangement, and bark patterns, are often unreliable due to environmental factors and phenotypic plasticity [15,16]. Misidentification in natural populations and cultivated specimens complicates conservation strategies and genetic resource management.

Molecular markers are highly informative for analyses of intra- and interspecific variation and can be determined regardless of phenotype, allowing for unbiased comparisons of adaptations, their genetic basis, and evolutionary processes [17]. Molecular markers have also emerged as powerful tools for resolving taxonomic ambiguities in closely related plant species. Among these markers, single nucleotide polymorphisms (SNPs) are particularly valuable owing to their high abundance and stability across genomes; they facilitate precise species identification and cultivar discrimination by detecting minor genetic variation [18,19]. The application of SNP markers has revolutionized genetic studies in forestry, enabling more accurate assessments of genetic diversity, population structure, and evolutionary relationships [20–22]. In the case of *Abies nordmanniana* (Nordmann fir), a large pool of SNPs was identified by exploiting a combination of approaches, including a significant number with potential downstream applications, including applications in pedigree reconstruction, clone identification, and genomic selection [23].

Despite the effectiveness of molecular marker methods, they often require complex laboratory procedures and specialized expertise, limiting their practical application in routine species identification. There is a need for a rapid, reliable, and user-friendly SNP detection system that can be easily adopted in various settings, including field conditions. To address this issue, various technologies have been developed for SNP detection, including Kompetitive Allele-Specific PCR (KASP) and Allele-Specific PCR (AS-PCR), each with its advantages in terms of sensitivity, throughput, and cost-effectiveness [19,24]. KASP is a homogeneous, fluorescence-based genotyping technology that allows for high-throughput and cost-efficient SNP analyses; it uses allele-specific primers and a FRET (Fluorescence Resonance Energy Transfer) quenching system to detect SNP variants, making it suitable for large-scale genotyping projects [19,25].

In this study, we developed a rapid and reliable species-specific DNA marker in the *nad5* intron 1 region of mitochondrial DNA to discriminate between *A. koreana* and *A. nephrolepis* using Competitive Allele-specific Labeled Light Emission (CALLE) technology [26]. Mitochondrial DNA is maternally inherited and shows low levels of heteroplasmy, making it a stable and reliable genetic marker for species differentiation [27]. Allele-specific primers were designed to target these SNPs, introducing intentional mismatches to enhance specificity. The marker combines allele-specific amplification with

labeled light emission, providing a straightforward and efficient method suitable for both laboratory and field applications.

2. Materials and Methods

2.1. Plant Materials and Sample Collection

The needles (leaves) of *A. koreana* and *A. nephrolepis* were selected as the plant materials for this study. These species were chosen based on their ecological significance and distinct geographical distributions within Korea. Samples of *A. koreana* were collected from Mt. Halla and outdoor fields of the National Institute of Ecology (NIE). *A. nephrolepis* samples were obtained from Mt. Seorak and outdoor fields of the NIE in Korea. Plants were collected from two sites per region. Detailed information about the collection sites, including geographic coordinates, is provided in Table 1. All collected samples were handled carefully and stored at -80°C to preserve their integrity. A subset of the samples was subsequently utilized for further experimental analyses.

Table 1. Geographic coordinates of *Abies koreana* and *Abies nephrolepis* sampling sites in Korea.

Taxon	Altitude	Sampling Site	Latitude	Longitude
<i>Abies koreana</i>	1663 m	Mt. Halla1	33.357904	126.5237
	1664 m	Mt. Halla2	33.357923	126.523713
	6 m	NIE1	36.037967	126.716567
	5 m	NIE2	36.038321	126.716565
<i>Abies nephrolepis</i>	1463 m	Mt. Seorak1	38.117005	128.401705
	1472 m	Mt. Seorak2	38.116951	128.401352
	6 m	NIE1	36.038287	126.716578
	6 m	NIE2	36.038335	126.716573

2.2. DNA Extraction

Genomic DNA was extracted from fresh leaves (100 mg) of *A. koreana* and *A. nephrolepis* using the Beniprep[®] Super Plant Total Nucleic Acid Extraction Kit (Cat# IVT7006, Invirustech, Gwangju, Republic of Korea) following the manufacturer's protocol, with minor modifications. Briefly, leaf samples were ground to a fine powder in liquid nitrogen and homogenized with 400 μL of PHB2A buffer containing 50 μL of nuclease destroyer (Cat# IN4001, Invirustech, Gwangju, Republic of Korea). After adding 450 μL of LYB buffer, the mixture was incubated at 55°C for 5 min. RNase A (1 μL , 1 mg/mL) was added to remove RNA contamination. The lysate was treated sequentially with 300 μL of SCP buffer and 400 μL of RLC buffer, with centrifugation steps ($\geq 15,000\times g$, 4°C) between treatments. The supernatant was loaded onto a binding column and washed successively with PW1, PW2, and PW3 buffers. Final DNA elution was performed with 50–100 μL of elution buffer. The quality and quantity of extracted DNA were assessed using a microplate spectrophotometer system (Biotek-Epoch, Winooski, VT, USA).

2.3. Selection of Candidate Genes and PCR Amplification

Candidate genes were identified based on a de novo RNA-seq assembly and comparative analysis. We have previously performed a transcriptomic analysis of *A. koreana* trees exposed to heat or drought stress for different time periods, respectively [28,29]. RNA-seq data provided insight into the genes related to the response to heat and drought stresses in *A. koreana*. In this study, we focused on genes whose expression increased by more than 2-fold under heat or drought stress. We isolated candidate genes using primers for *A. koreana* in *A. nephrolepis*. Four candidate genes displayed sequence differences between *A. koreana* and *A. nephrolepis*. In addition, mitochondrial DNAs of *nad5* intron 1 and *nad5* intron 4 were included as candidate genes for comparisons of nucleotide sequences between

A. koreana and *A. nephrolepis* (NCBI GenBank accession No.; KT448846-KT449415) [15], for a total of six genes included in analyses.

Two genes showing differential expression in *A. koreana* following high-temperature treatment [28] and two genes showing differential expression after drought treatment [29] were selected. Candidate genes were evaluated for sequence differences between *A. nephrolepis* and *A. koreana*. The primers were designed with varying sizes to amplify variable regions of the genes, as shown in Table 2. In addition, primers targeting the *nad5* intron 1 and intron 4 regions of mitochondrial DNA were used [15,30,31] (Table 2). PCR amplification was performed using BioFACT™ A-Star Taq DNA Polymerase (AT116-500, Biofact Co., Daejeon, Republic of Korea) with the following thermal cycling conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at (T_m: 58 or 60 °C) for 30 s, and extension at 72 °C for 30 s. The PCR products were analyzed by electrophoresis on 1.5% agarose gels in 0.5× TBE buffer at 150 V for 30 min. Three microliters of each PCR product were loaded onto the gel, and the DNA bands were visualized under UV light after ethidium bromide staining. In addition, PCR products for the selected target genes were purified and subjected to Sanger sequencing with the bidirectional sequencing and quality-control procedure (ABI 3730XL, BigDye® Terminator v3.1 Cycle Sequencing Kit) (Bionics Co., Seoul, Republic of Korea). The obtained sequences were analyzed using MAFFT (Multiple Alignment using Fast Fourier Transform) software (version 7) [32]; in particular, a multiple sequence alignment was generated to identify species-specific variants. Sequence alignment files were visualized and analyzed using BioEdit software (version 7.2) to identify diagnostic SNPs.

Table 2. Primers used in this study.

Gene	Direction	Sequence (5′ → 3′)	Size (bp)	T _m (°C)	Reference
<i>nad5</i> intron 1	Forward	GGAAATGTTTGATGCTTCTTGGG	1259	58	[15]
	Reverse	CTGATCCAAAATCACCTACTCG			
<i>nad5</i> intron 4	Forward	CATCCCTCCCATTGCATTAT	302	58	[15]
	Reverse	GGACAATGACGATCCGAGATA			
<i>AkAnGCR2</i>	Forward	GCCTGAACTGTATTTCGTCAATAATC	556	60	[29]
	Reverse	CAGCCCATAGAAATCCTGCAC			
<i>AkAnMYB123</i>	Forward	CCGGAAGAAGAGGAGCTCG	532	60	[28]
	Reverse	CCTGCAATGTTTTACTTGGACTG			
<i>AkAnEFP</i>	Forward	GCGCTGGACAAAGATCACAATG	320	60	[28]
	Reverse	GCGAACGCATCGATTTCAAAAG			
<i>AkAnATL78</i>	Forward	GCCGTCTGTTTGAATTCATG	220	60	[29]
	Reverse	CTTCGGCGAACTCGGAGAG			

2.4. Construction of a Pipeline for Species-Specific Marker Development

A pipeline for species-specific marker development was used to design allele-specific primers automatically using the M2011 web tool [26]. SNP positions and allelic variants were identified from DNA sequences, and Primer3 was used for primer design. Forward primers were optimized for T_m (55–60 °C) with mismatches introduced for allele specificity. Reverse primers were designed downstream of the SNP (T_m: 61–68 °C), and amplicon sizes were limited to 300 bp. The automated pipeline was made publicly available via a web-based tool, ASG Designer 2 (<https://asgdesigner2.ivttools.com>, accessed on 21 July 2026). Experimental validation via real-time PCR confirmed the specificity and efficiency, enabling rapid SNP genotyping.

2.5. SNP Genotyping Using a Species-Specific Marker

The species-specific marker was used for rapid SNP-based differentiation of *A. koreana* and *A. nephrolepis*. PCRs (10 µL) were prepared using 2× master mix, primer mix, Q-FRET mix_V1 and 2 µL of DNA template (>10 ng/µL). The qRT-PCR was run under the following

conditions: initial denaturation at 95 °C for 2 min, followed by 10 cycles of 94 °C for 10 s and 56 °C for 30 s and then 26 cycles of 94 °C for 10 s, 61 °C for 30 s and 40 °C for 15 s. Fluorescent signals were read at 40 °C for allelic discrimination using FAM (*A. koreana*) and HEX (*A. nephrolepis*) dyes. Results were analyzed using the QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with QuantStudio™ Design & Analysis Desktop Software v1.6.1 and the CFX Opus Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA) with CFX Maestro Software v2.3 (build 5.3.002.1030) real-time PCR platforms.

3. Results

3.1. Sampling Sites of *A. koreana* and *A. nephrolepis* in Korea

A. koreana and *A. nephrolepis* are coniferous trees that grow in temperate and subarctic regions of the Northern Hemisphere, including Korea. In this study, samples were collected from Mts. Halla, Seorak and an exhibition area of the NIE in Korea. Mt. Halla, located in the southernmost part of the Korean Peninsula, has an elevation of 1950 m above sea level, making it the tallest mountain in South Korea. It is also the region with the highest density of *A. koreana* habitats [16]. Mt. Seorak, located in the northeastern region of South Korea, is one of the country's most iconic mountains and harbors the largest colony of *A. nephrolepis* in South Korea [10]. The plants in each region were sampled and geographic coordinates were recorded (Table 1).

3.2. Amplification of Candidate Genes for a Species-Specific Marker Distinguishing *A. koreana* and *A. nephrolepis*

We performed electrophoresis and found that the amplification patterns showed no difference between *A. koreana* and *A. nephrolepis* (Figure 1). The single band was detected in *nad5* intron 1 (Figure 1A). For *nad5* intron 4, the product exhibited multiple bands, including a faint band in lane 4, and the bands were successfully produced in all samples (Figure 1B). The *AkAnGCR2* gene was not amplified in any sample, and *AkAnMYB123* showed multiple nonspecific bands (Figure 1C,D). *AkAnEFP* and *AkAnATL78* clearly showed a single amplification product (Figure 1E,F). Finally, three loci, *nad5* intron 1, *AkAnEFP*, and *AkAnATL78*, were selected for a DNA sequencing analyses.

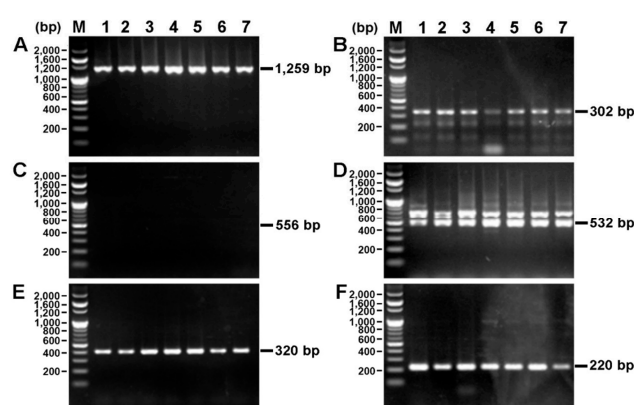


Figure 1. PCR amplification from *Abies koreana* and *Abies nephrolepis* samples. Lane M: 100 bp DNA Ladder (Enzynomics, Daejeon, Republic of Korea); Lanes 1–2: *A. koreana* samples from the National Institute of Ecology; Lanes 3–4: *A. koreana* samples from Mt. Halla; Lanes 5–6: *A. nephrolepis* samples from the National Institute of Ecology; Lane 7: *A. nephrolepis* sample from Mt. Seorak. PCR products were separated on 1.5% agarose gels at 150 V for 40 min. Panels: (A) *nad5* intron 1 (~1259 bp), (B) *nad5* intron 4 (302 bp), (C) *AkAnGCR2* (556 bp), (D) *AkAnMYB123* (532 bp), (E) *AkAnEFP* (320 bp), (F) *AkAnATL78* (220 bp). The experiments were independently conducted in triplicate, and a representative image is shown.

3.3. SNPs in *nad5* Intron 1 of *A. koreana* and *A. nephrolepis*

SNPs are one of the most prevalent forms of genetic variation, providing valuable information for species identification, phylogenetic studies, and evolutionary analyses. To evaluate SNPs, nucleotide sequence alignments of *nad5* intron 1, *AkAnEFP*, and *AkAnATL78* were generated for *A. koreana* and *A. nephrolepis*. Neither *AkAnEFP* nor *AkAnATL78* showed sufficient sequence variation to differentiate between the two species. Interestingly, there were distinct SNPs between *A. koreana* and *A. nephrolepis* in *nad5* intron 1.

The mitochondrial *nad5* gene encodes a subunit of NADH dehydrogenase subunit5, a crucial part of complex I, which is the first and largest enzyme in the electron transport chain and plays an essential role in plant respiration and electron transport [33]. In addition, intronic regions, though non-coding, often contain conserved sequences and SNPs that can be useful for genetic differentiation. Therefore, the SNP sites in *nad5* intron 1 at positions 530869 and 530938 were evaluated in each species using DNA samples from Mts. Halla, Seorak and the exhibition area of the NIE in Korea (Figure 2). Specifically, a T-to-G mutation was observed, with *A. koreana* exhibiting a T at the variant position 530904 and *A. nephrolepis* showing a G. These results suggest that *nad5* intron 1 could serve as a candidate gene for a species-specific marker.

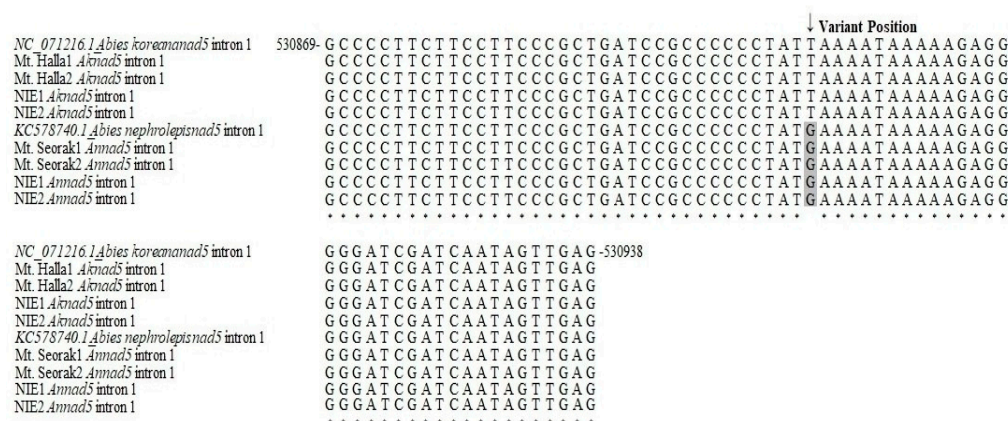


Figure 2. Validation of accession-specific single nucleotide polymorphisms (SNPs) in *nad5* intron 1 regions of *Abies koreana* and *Abies nephrolepis* mitochondrial DNA. *nad5* intron 1 region sequences of *A. koreana* and *A. nephrolepis* were compared to identify species-specific SNPs. Conserved and variable nucleotide positions are shown in the alignment. A variant position is indicated with arrow, showing clear differentiation between *A. koreana* (NC_071216.1 and related sequences) and *A. nephrolepis* (KC578740.1 and related sequences). SNPs unique to each species are marked at positions 530869 and 530938. Asterisks (*) indicate conserved nucleotide positions across all accessions. The results were validated through resequencing, confirming the presence of species-specific SNPs critical for genetic identification and differentiation of the two species. The alignment is based on Sanger-derived consensus sequences.

3.4. Development of *nad5* Intron 1 as a Marker for Species Differentiation Between *A. koreana* and *A. nephrolepis*

To develop a species-specific marker using *nad5* intron 1 for *A. koreana* and *A. nephrolepis*, we designed two allele-specific primers to detect single nucleotide variants (SNVs). The primers selectively amplified the T and G alleles, enabling effective differentiation between the two species (Table 3). The FAM_ *AkAnnad5* intron 1_T primer was designed to detect the T allele-specific sequence in *A. koreana*. An intentional mismatch (T → C) was introduced at the 3' end of the forward primer to enhance specificity. This primer was labeled with the FAM fluorophore for detection of the T allele. The HEX_ *AkAnnad5* intron 1_G primer was designed to detect the G allele-specific sequence in *A. nephrolepis*. Similarly, an intentional mismatch (T → C) was introduced at the 3' end to increase marker specificity. This primer

was labeled with the HEX fluorophore for the detection of the G allele. In addition, the *Rev_AkAnnad5* intron 1 primer served as a universal reverse primer, amplifying the *nad5* intron 1 region in both species. These primers could accurately detect species-specific SNVs, and the introduction of intentional mismatches at the 3' ends of the primers improves specificity (Supplementary Figure S1).

Table 3. Allele-specific primers for *nad5* intron 1 in *Abies koreana* and *Abies nephrolepis*.

Primer	Sequence (5' → 3')	Label	Length (bp)	T _m (°C)	Function
<i>FAM_AkAnnad5</i> intron 1_T	TAG1- ATCGATCCCCCTCTTTTATTC*TA	FAM	24	55.5	Detects T allele (<i>A. koreana</i>)
<i>HEX_AkAnnad5</i> intron 1_G	TAG-2- TCGATCCCCCTCTTTTATTC*TC	HEX	23	56.2	Detects G allele (<i>A. nephrolepis</i>)
<i>Rev_AkAnnad5</i> intron 1	CTCCTTCCCGCTGATCCGC	Common Reverse	20	64.5	Common reverse primer for amplification

Notes: The asterisk (*) in the forward primer sequences indicates an intentional mismatch introduced to increase specificity. This mismatch is located at the third position from the 3' end (T → C). Proprietary sequences (TAG1 and TAG2) are internal technical details and used for performance optimization. This table provides details of allele-specific primers designed for detecting single nucleotide variations (SNVs) in the *nad5* intron 1 region of *A. koreana* and *A. nephrolepis*. The *FAM_AkAnnad5* intron 1_T primer identifies the T allele specific to *A. koreana*, while *HEX_AkAnnad5* intron 1_G detects the G allele specific to *A. nephrolepis*. The *Rev_AkAnnad5* intron 1 primer serves as a universal reverse primer.

Finally, these primers were validated using real-time PCR systems, including the ABI QuantStudio 3 and Bio-Rad CFX Opus platforms (Figure 3). For ABI QuantStudio 3, fourteen samples (six *A. koreana* and eight *A. nephrolepis*) were tested in triplicate. Fluorescence data, normalized to the ROX passive reference dye, confirmed clear separation of species-specific alleles. The FAM signal (Allele 1) corresponded to *A. koreana*, and the HEX signal (Allele 2) corresponded to *A. nephrolepis* (Figure 3A). In addition, the Bio-Rad CFX Opus system, using post-amplification fluorescence analysis at 40 °C without a passive reference dye, confirmed species discrimination using four samples (two *A. koreana* and two *A. nephrolepis*) with three replications (Figure 3B). Both platforms showed clear differentiation of T (FAM) and G (HEX) alleles, consistent with the maternal inheritance of mitochondrial DNA and absence of heteroplasmy. Therefore, these results strongly demonstrate the establishment of a rapid and robust diagnostic method for distinguishing *A. koreana* and *A. nephrolepis*.

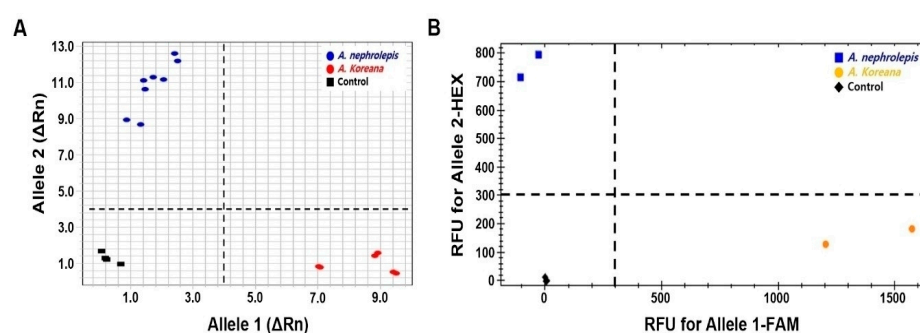


Figure 3. Allelic discrimination plots for species-specific *nad5* intron 1 SNP markers in *Abies koreana* and *Abies nephrolepis*. (A) Results obtained using the ABI QuantStudio 3 system, utilizing pre-amplification and post-amplification fluorescence readings normalized with ROX passive reference dye. SNP discrimination is based on the relative fluorescence signal intensity for FAM (T allele) and HEX (G allele). Each sample was analyzed in triplicate. (B) Results obtained using the Bio-Rad CFX Opus system, without a passive reference dye. SNP discrimination was performed by lowering the temperature to 40 °C post-amplification to monitor FRET-based fluorescence generation or quenching, yielding CT values for allele assignment. Both methods effectively differentiated *A. koreana* (FAM signal) and *A. nephrolepis* (HEX signal). The experiments were independently conducted in triplicate, and a representative image is shown. The dotted lines indicate threshold determination.

4. Discussion

Since the late 1990s, the increasing frequency of high temperatures and drought conditions due to global climate change has contributed to substantial forest mortality and tree dieback worldwide [34]. Widespread conifer mortality due to recurring droughts since the 1970s has been observed, affecting up to 20% of coniferous forests in Switzerland, France, Poland, and Greece in Europe. In North America, there are increasing reports of extensive conifer dieback due to drought, particularly in the western United States, affecting pine, fir, and spruce forests across vast areas. In addition, extensive conifer mortality due to drought has been observed in China, Russia, and other regions. Extreme drought has led to the decline in *A. koreana* (20–50%) in South Korea. Alpine coniferous forests, in particular, have experienced severe declines, necessitating comprehensive research on the causes and conservation trends at a global scale. Understanding these patterns is crucial for establishing directions for conservation research focused on alpine vulnerable ecosystems in South Korea.

The genus *Abies* includes several coniferous species distributed across the Northern Hemisphere. Among them, *A. koreana* and *A. nephrolepis* are significant species in East Asia. Understanding their distinct characteristics and conservation requirements is essential for maintaining biodiversity and ecosystem stability [13,35]. *A. nephrolepis* has a relatively wide range; however, *A. koreana* requires immediate intervention owing to its endangered status. A combined effort involving habitat protection, research, and public engagement is necessary to safeguard these valuable species for future generations [13,36]. *A. koreana* and *A. nephrolepis* are both subalpine conifer species found in South Korea; however, their distribution and climate adaptability differ significantly (Table 1). *A. koreana* is endemic to South Korea, primarily inhabiting subalpine regions, such as Mts. Halla, Jiri, and Deogyu at elevations of 1000 to 1900 m. It thrives in cool-temperate to subalpine climates but is highly vulnerable to climate change, which has led to severe population decline and habitat loss [3]. By contrast, *A. nephrolepis* has a broader distribution, spanning northern and central Korea, Russia, China, and Japan. It grows at 1200 to 2000 m in subalpine to boreal climates and is more resilient to colder temperatures. While the species is less affected by climate change compared with *A. koreana*, habitat destruction and warming trends could still pose risks [35].

It is essential to distinguish *A. koreana* and *A. nephrolepis* using molecular ecological methods. Traditional identification based on physical characteristics can be unreliable, especially in hybrid zones or areas where environmental variations affect phenotypic traits [37]. Therefore, molecular markers, such as chloroplast DNA (cpDNA) sequences, microsatellites (SSRs), and SNPs, could provide a precise and reproducible means of differentiation. These genetic tools enable accurate species identification, help in understanding evolutionary relationships, and support conservation efforts by identifying genetic diversity and potential threats, such as habitat loss or climate change. To develop a species-specific marker for *A. koreana* and *A. nephrolepis*, we evaluated four candidate genes related to the response to heat or drought stress in addition to mitochondrial *nad5* intron 1 and 4 (Table 2). Finally, *nad5* intron 1 was identified as a marker for differentiation between *A. koreana* and *A. nephrolepis* (Figure 2).

The mitochondrial *nad5* gene (NADH dehydrogenase subunit5) encodes a subunit of NADH:ubiquinone oxidoreductase, also known as Complex I of the mitochondrial respiratory chain, which functions as an electron transport chain component and in ATP production and energy metabolism [38]. Given its role in the electron transport chain, mutations or impairments in *nad5* can affect metabolic processes and lead to various mitochondrial diseases [38]. Variation in intronic regions of the *nad5* gene could be species-specific [31,39], and several properties of *nad5* make it an ideal marker for distinguishing

between *A. koreana* and *A. nephrolepis*. For example, mitochondrial DNA is maternally inherited and is less prone to genetic variation caused by hybridization. Furthermore, because mitochondrial DNA is haploid and lacks heterozygous phenotypes, the *nad5* intron 1 region provides a stable and consistent marker (Figure 2). Low heteroplasmy and the predictable inheritance of alleles also make this region a robust choice for molecular identification.

Finally, we developed a marker for differentiation between *A. koreana* and *A. nephrolepis* using allele-specific primers for *nad5* intron 1 (Table 3). Real-time PCR technology enables rapid, precise quantification and clear allelic discrimination. The primers were validated using real-time PCR platforms (i.e., the ABI QuantStudio 3 and Bio-Rad CFX Opus systems) (Figure 3), demonstrating the utility of the developed primers for rapid species identification. The fluorescence signals corresponding to species-specific alleles facilitated clear discrimination, without the need for complex procedures or extensive equipment, making the method both efficient and practical. By offering a rapid and user-friendly method for distinguishing between *A. koreana* and *A. nephrolepis*, the marker could play a vital role in improving seedling authenticity and preventing misidentification in both conservation and commercial contexts.

Thus, the *nad5* intron 1 region represents a robust genetic marker for differentiation between *A. koreana* and *A. nephrolepis*. The marker offers a practical and efficient tool, addressing critical needs in conservation, forestry management, and commercial applications. By enabling accurate and rapid identification, the marker supports the integrity of breeding programs, promotes sustainable utilization, and contributes to the conservation of these economically and ecologically significant species. Moreover, integrating additional SNP markers into the diagnostic pipeline will strengthen the reliability of the method and expand its applicability. These expanded markers could be used to generate detailed distribution maps for *A. koreana* and *A. nephrolepis*, which would serve as valuable tools for conservation planning and monitoring the effects of environmental changes on species distributions. It could be also instrumental in understanding how climate change, land-use changes, and other ecological factors affect the distribution and population dynamics of these species.

5. Conclusions

We developed a rapid and reliable SNP-based diagnostic method for distinguishing *A. koreana* and *A. nephrolepis* using the *nad5* intron 1 region, despite the limited genomic resources available for these species. Leveraging the stability and maternal inheritance of mitochondrial DNA, the marker enables accurate species identification using allele-specific primers and real-time PCR. Compared with traditional methods, this approach is simpler, faster, and more accessible, making it highly applicable for forestry management, conservation, and commercial use. While the current research was limited to a small sample set, the results demonstrate the potential of molecular markers in resolving taxonomic ambiguities, even in species with limited genomic data. Further validation and expansion of SNP markers will enhance the practical utility of the approach and enable broader applications.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/conservation6030090/s1>, Figure S1: ROC curve of the ASQ genotyping assay for discriminating *Abies koreana* from *Abies nephrolepis* ($n = 6$ and $n = 8$, respectively). The assay showed perfect separation of the two species (AUC = 1.000). The dashed diagonal indicates random classification (AUC = 0.5).

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Data Availability Statement: The authors declare that the data collected for this project can be shared with other scientists.

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