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DNA Barcodes for Wood Identification of Anatomically Similar Species of Genus *Chamaecyparis*

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Abstract: The genus *Chamaecyparis* comprises seven species (*C. formosensis*, *C. hodginsii*, *C. lawsoniana*, *C. nootkatensis* (*Callitropsis nootkatensis*), *C. obtusa*, *C. pisifera*, and *C. thyoides*). Accurate species identification is necessary for proper use and economic value of wood. Species identification of woods is generally based on anatomical analysis; however, *C. obtusa* and *C. pisifera* wood have similar microscopic morphology, which makes species identification impossible. Therefore, the molecular identification of species in wood of the genus *Chamaecyparis* is required. In this study, six candidate DNA barcode genes (*trnP-GGG*, *ycf1*b, *clpP*, *accD*, *ycf2*, and *rps16*) in the chloroplast of *Chamaecyparis* were identified with nucleotide diversity values higher than the arbitrary value of 0.02. Each gene was evaluated for species identification using phylogenetic analysis by genes registered at NCBI (42 sequences each for *trnP-GGG*, *ycf1*b, *clpP*, *accD*, and *ycf2*, and 50 sequences for *rps16*). The genes *trnP-GGG*, *clpP*, and *rps16* could not be distinguished between *C. pisifera* and *C. formosensis*. However, *ycf1*b, *accD*, and *ycf2* could be distinguished between all *Chamaecyparis* species. These results suggest the use of the chloroplast genes *ycf1*b, *accD*, and *ycf2* as DNA barcodes for species identification in *Chamaecyparis*, including *C. obtusa* and *C. pisifera*, based on the reported genetic information to date.

Keywords: wood species identification; DNA barcode; *Chamaecyparis*; nucleotide diversity; phylogenetic analysis

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1. Introduction

A survey in 2012 found that 15–30% of forests were being illegally logged worldwide [1]. Unplanned logging causes landslides and flooding, which adversely affect the safety and economy of local communities [2]. Illegal logging also adversely affects the environment, as forests serve as carbon reservoirs as well as natural habitats for many animals and plants [2,3]. According to the 2006 World Bank data, illegal logging causes an estimated annual loss of USD 1.5 billion, with losses in the legal forest industry accounting for more than 60% of the total [3,4]. Many countries, including the United States, Australia, and countries in Europe, have banned the import and trade of illegal timber [5,6]. Accurate wood identification is critical for the successful enforcement of regulations against the illegal timber trade [6]. Wood identification can also help protect forests by controlling the trade of wood obtained from endangered species or forests in need of protection [6]. DNA barcoding for timber species identification can authenticate their origin [7]. The Forest Stewardship Council (FSC), which tracks and manages the harvesting, processing, and distribution of timber, argues that tracing the origin of timber helps to prevent illegal logging and can also help to ensure a sustainable forest [8,9]. Consumers can access wood based on species-specific characteristics by using wood identification [10].

The genus *Chamaecyparis* is mainly found in East Asia and North America and their wood is used as high-end building and furniture materials [11–13]. *Chamaecyparis* comprises seven species, including *C. formosensis*, *C. hodginsii*, *C. lawsoniana*, *Callitropsis nootkatensis* (homotypic synonym: *C. nootkatensis*), *C. obtusa*, *C. pisifera*, and *C. thyoides* [14,15]. The mixed forests of subtropical eastern Asia are inhabited by the genus *Chamaecyparis*, especially *C. lawsoniana* [11,16]. *C. formosensis* is used for furniture due to its high wood quality, aroma, and durability [17]. *C. lawsoniana* is widely planted for landscaping in North America and Europe, where it has ecological and economical value [18]. Extracts of *C. obtusa* are used in medicinal preparations for their antifungal and anti-inflammatory properties [19,20]. *C. formosensis* and *C. obtusa* are threatened by illegal logging in Taiwan [21,22]. The conservation statuses of *C. formosensis*, *C. hodginsii*, *C. lawsoniana*, and *C. obtusa* are endangered, vulnerable, near threatened, and near threatened, respectively (https://threatenedconifers.rbge.org.uk/taxonomy/cupressaceae/p2 (accessed on 25 February 2024)). *C. lawsoniana* is found in a limited region and requires protection from logging and root rot disease [11]. Therefore, each species of *Chamaecyparis* has different reasons for accurate species identification, depending on the situation.

Wood species are primarily identified by observing anatomical features under a microscope [4,5,23–26]. However, *C. obtusa* and *C. pisifera* have similar pit numbers, pit size, pit type, ray frequency, and ray height, making it difficult to distinguish them based on anatomical characteristics alone [27]. DNA barcoding technology is an alternative method that can be used to distinguish anatomically similar species [28,29]. Using genes from chloroplasts to identify wood species has many advantages. The presence of a large number of identical chloroplasts in a single cell makes it easier to obtain analyzable chloroplast genes from old, processed wood. In addition, chloroplast DNA multiplies by binary fission and is inherited only from the maternal line, so there is no mixing of genes through fertilization, providing genes that can be used for species identification. Previous studies showed that phylogenetic analysis of the genus *Chamaecyparis* using *matK*, which is a chloroplast DNA barcode commonly used to distinguish plants, failed to distinguish between *C. formosensis* and *C. pisifera* [30]. DNA barcoding using the internal transcribed spacer region, which is generally used to classify eukaryotic cells, also failed to distinguish between *C. lawsoniana* and *C. obtusa* [31,32]. Further, *petG-trnP* and *trnV* cannot be used to distinguish between *C. formosensis*, *C. lawsoniana*, and *C. obtusa* [14].

In the present study, we propose new DNA barcodes that can accurately identify all seven species of *Chamaecyparis,* including species that are anatomically indistinguishable. For this purpose, all complete chloroplast genome sequences of the genus *Chamaecyparis* were obtained from the National Center for Biotechnology Information (NCBI), and genes with high variation were selected. A phylogenetic analysis of the selected genes was performed to evaluate their functionality as DNA barcodes. Accurate identification of all species in the genus *Chamaecyparis* provides an opportunity for the many benefits mentioned above.

2. Materials and Methods

2.1. Measurement of Nucleotide Diversity

The nine chloroplast genomes (NCBI accession numbers: LC522362 (*C. formosensis*), NC_034943 (*C. formosensis*), KX832623 (*C. hodginsii*), MG269834 (*C. hodginsii*), KX832622 (*C. lawsoniana*), LC529363 (*C. obtuse*), MT258872 (*C. obtusa*), MT334621 (*C. pisifera*), and NC_057503 (*C. pisifera*)), for which complete sequences have been reported in the genus *Chamaecyparis*, were collected from the NCBI database and aligned using the ClustalW program [33]. Nucleotide diversity was calculated using the DnaSP (ver. 6.0) software with 100 bp of window length and 50 bp of step size [34].

2.2. Gene Alignment and Phylogenetic Analysis

Gene alignments and phylogenetic analysis were performed as described in our previous study [35]. Briefly, gene sequences of *accD*, *clpP*, *trnP-GGG*, *ycf1*b, *ycf2*, and *rps16*, with a query coverage of more than 90% for the genus *Chamaecyparis*, were collected and aligned using the ClustalW program [33]. Forty-two genes for each *accD*, *clpP*, *trnP-GGG*, *ycf1*b, and *ycf2*, and fifty genes for *rps16* were obtained from the NCBI database. The NCBI accession numbers of the genes used are listed in each Figures, showing the results of the phylogenetic analysis, and in the Supplementary Material Figures. Phylogenetic analysis was performed with 1000 bootstraps using the Kimura two-parameter model in the MEGA (ver. 11.0) software using the maximum likelihood method [36]. *trnP-GGG* from *Juniperus chinensis*; *ycf1*b, *clpP*, *accD*, and *ycf2* from *Thuja occidentalis*; and *rps16* from *Calocedrus formosana* were used as an outgroup. Alignment results were displayed using BioEdit (ver. 7.7.1) to present the sequence alignment [37].

3. Results and Discussion

3.1. Evaluation of Nucleotide Diversity of Chloroplast Genomes within Chamaecyparis

The nucleotide diversity of each gene was assessed using the DnaSP (ver. 6.0) software with the nine reported complete chloroplast genomes of the genus *Chamaecyparis* (NCBI accession numbers: LC522362 (*C. formosensis*), NC_034943 (*C. formosensis*), KX832623 (*C. hodginsii*), MG269834 (*C. hodginsii*), KX832622 (*C. lawsoniana*), LC529363 (*C. obtuse*), MT258872 (*C. obtusa*), MT334621 (*C. pisifera*), and NC_057503 (*C. pisifera*)) (Figure 1). Intergenic spacer sequences can exhibit high variation due to inversions and insertions within sequences of the same species, making species identification difficult [38]. Therefore, the nucleotide diversity of all genes in the chloroplasts was evaluated. Nucleotide diversity is an indicator of the genetic variation in a gene across species, and genes with high diversity can be used as DNA barcodes [39]. Six genes, *trnP-GGG*, *ycf1*, *clpP*, *accD*, *ycf2*, and *rps16*, with arbitrary nucleotide diversity (pi) values > 0.02 were selected for functional evaluation as DNA barcodes.

trnP-GGG, a transfer RNA coding for GGG to glycine [40], had the highest pi value. A previous study similarly reported that *trnP-GGG* in the Cupressaceae family has a high pi value [41]. *ycf1*, which had the second-highest pi value, often shows a high pi value among species and has been used in previous studies to identify members of the genus *Pinus* [42] and the family Orchidaceae [43]. In the genus *Chamaecyparis*, *ycf1* is a long gene with a nucleotide sequence of approximately 7000 bp, which exceeds the optimal length for use as a DNA barcode. Therefore, we used one of the small regions of *ycf1*, *ycf1b*, which has previously shown high efficiency as a DNA barcode [44]. *clpP* encodes a subunit of ATP-dependent protease [45]. *clpP* has been proposed as a DNA barcode for 27 species of the family Actinidiaceae; however, it cannot accurately distinguish all species [46]. *accD* encodes the acetyl-CoA carboxylase subunit D [47]. *accD* is used in combination with other genes to classify the genus Hexachlamys; however, two of the four species cannot be accurately identified [48]. *ycf2*, which encodes an FstH-like protein [49], shows the second-highest gene diversity among eight genes (*matK*, *rbcL*, *rpl20-rps18*, *trnH-psbA*, *trnL-trnF*, *trnV*, *ycf1*, and *ycf2*) within the genus *Pinus*, thereby demonstrating its potential as a DNA barcode [50]. *rps16* encodes a ribosomal protein [51], and its functionality as a DNA barcode for identifying species of the genus *Bupleurum* was evaluated in conjunction with an internally transcribed spacer; however, it cannot accurately identify species [52].

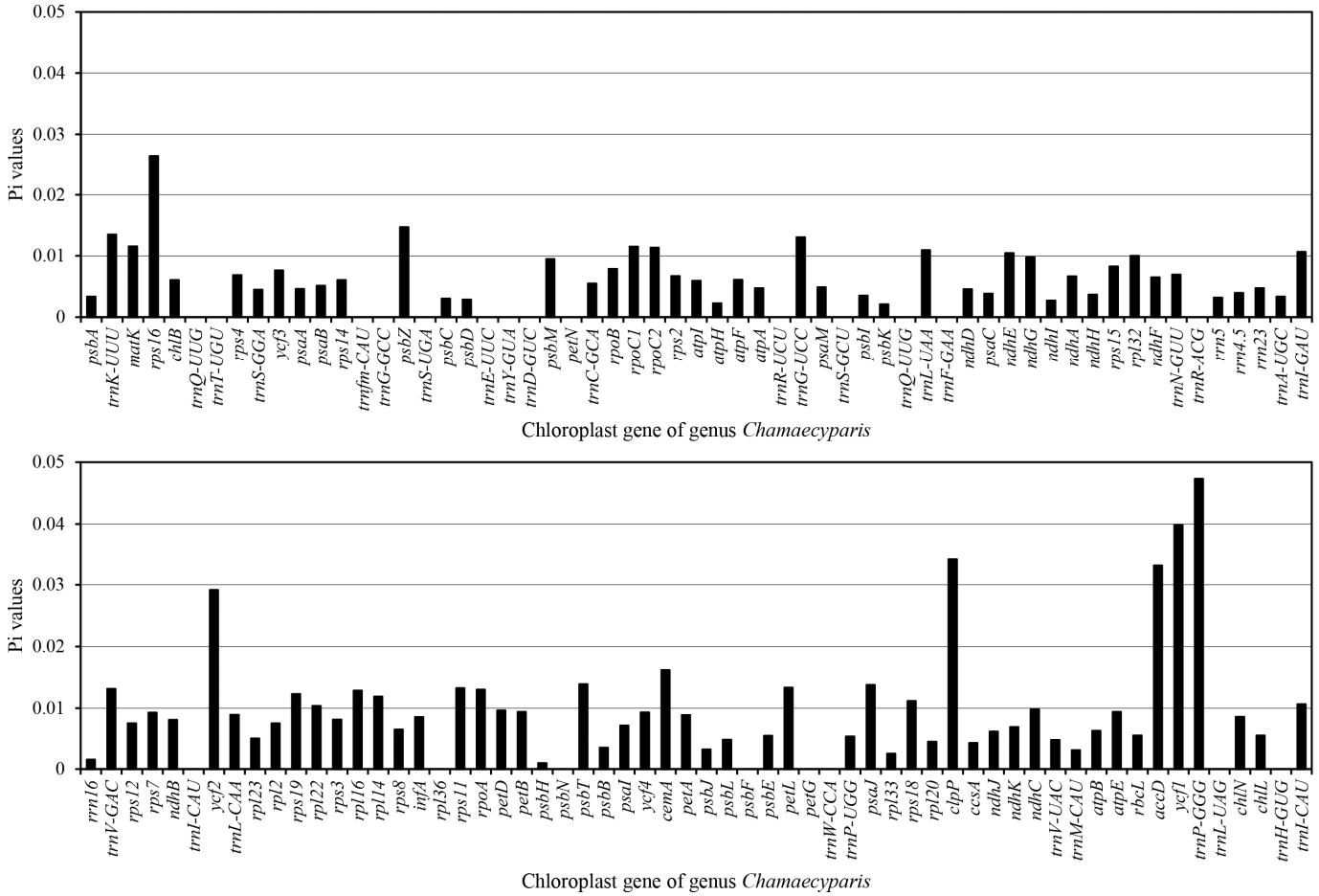

Figure 1. Analysis of nucleotide diversity values of genes in nine complete chloroplast genomes of the genus *Chamaecyparis* (*C. formosensis* (LC522362, NC_034943), *C. hodginsii* (KX832623, MG269834), *C. lawsoniana* (KX832622), *C. obtusa* (LC529363, MT258872), and *C. pisifera* (MT334621, NC_057503)) using DnaSP (ver. 6.0) software. Pi, nucleotide diversity value. Due to the large number of genes, it is presented in two graphs.

3.2. Phylogenetic Analysis of Five Species of Chamaecyparis *Using* trnP-GGG

trnP-GGG sequences of only five *Chamaecyparis* species (*C. formosensis*, *C. hodginsii*, *C. lawsoniana*, *C. obtusa*, and *C. pisifera*) are available in the NCBI database. All 42 reported *trnP-GGG* sequences from the genus *Chamaecyparis*, including genes from the complete chloroplast genome presented in Figure 1, were used to evaluate their functionality as a DNA barcode for species identification (Figure 2). Phylogenetic analysis could not accurately distinguish between *C. formosensis* and *C. pisifera*. When the gene sequences were aligned (Supplementary Material Figure S1), there were seven mismatches out of 72 bases. *trnP-GGG* showed the highest nucleotide diversity among chloroplast genes belonging to *Chamaecyparis*. However, the *trnP-GGG* genes of two indistinguishable species (*C. formosensis* and *C. pisifera*) had identical nucleotide sequences. A previous study suggested that many mutations in *trnP-GGG* of *Tortula ruralis* and *Physcomitrella patens* suggest the possibility of a pseudogene [53].

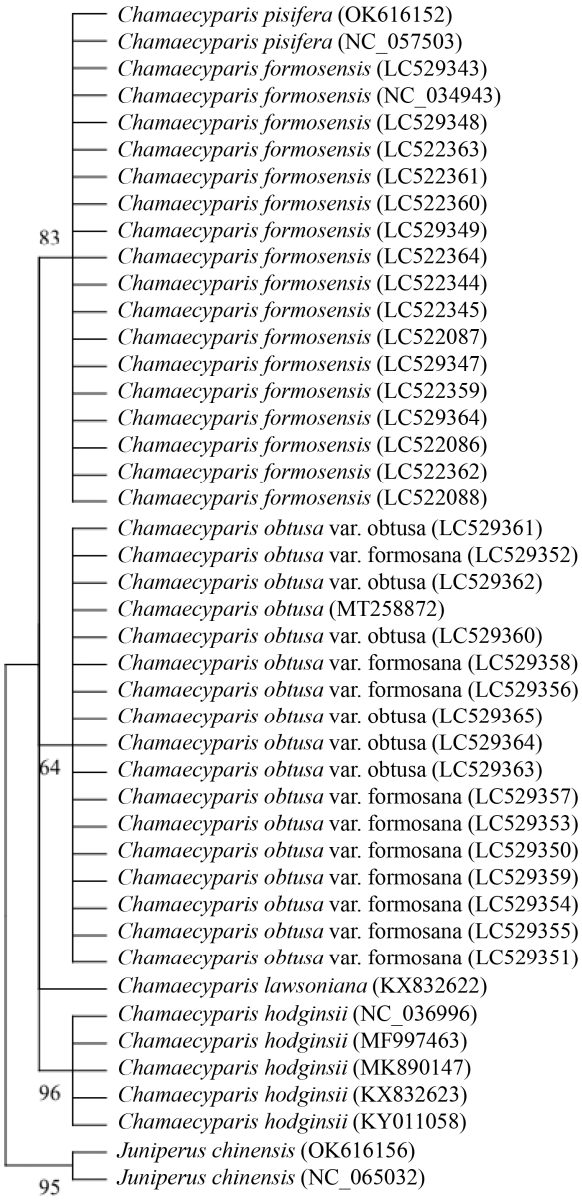

Figure 2. Phylogenetic analysis of five *Chamaecyparis* species (*C. formosensis*, *C. hodginsii*, *C. lawsoniana*, *C. obtusa*, and *C. pisifera*) using *trnP-GGG*. Phylogenetic analysis was performed with 1000 bootstraps using the Kimura two-parameter model in the MEGA (ver. 11.0) software using the maximum likelihood method.

3.3. Phylogenetic Analysis of Five Species of Chamaecyparis Using ycf1*b*

We investigated whether *ycf1*b can classify five species of *Chamaecyparis* (*C. formosensis*, *C. hodginsii*, *C. lawsoniana*, *C. obtusa*, and *C. pisifera*) based on phylogenetic analysis (Figure 3). In addition to the nine reported complete chloroplast genes presented in Figure 1, an additional thirty-three *ycf1*b sequences were used. Since all five species form an independent lineage group, *ycf1*b can be used as a DNA barcode for species identification in the genus *Chamaecyparis*. The length of the aligned *ycf1*b nucleotide sequence was 1469 bases, and the number of non-identical nucleotide sequences was 130 bases (Supplementary Material Figure S2). *ycf1*b could be used to distinguish between *C. formosensis* and *C. pisifera*. *ycf1*b can be used to identify 71.87% of 391 land plant species [54]. In the present study, *ycf1*b could also be used to accurately identify species of *Chamaecyparis*. Since the sequence of *ycf1*b for *C. nootkatensis* was not available, it was not included in this

evaluation. *ycf1*b could be used to distinguish *C. obtusa* from other species; however, it could not be used to distinguish the subspecies of *C. obtusa*.

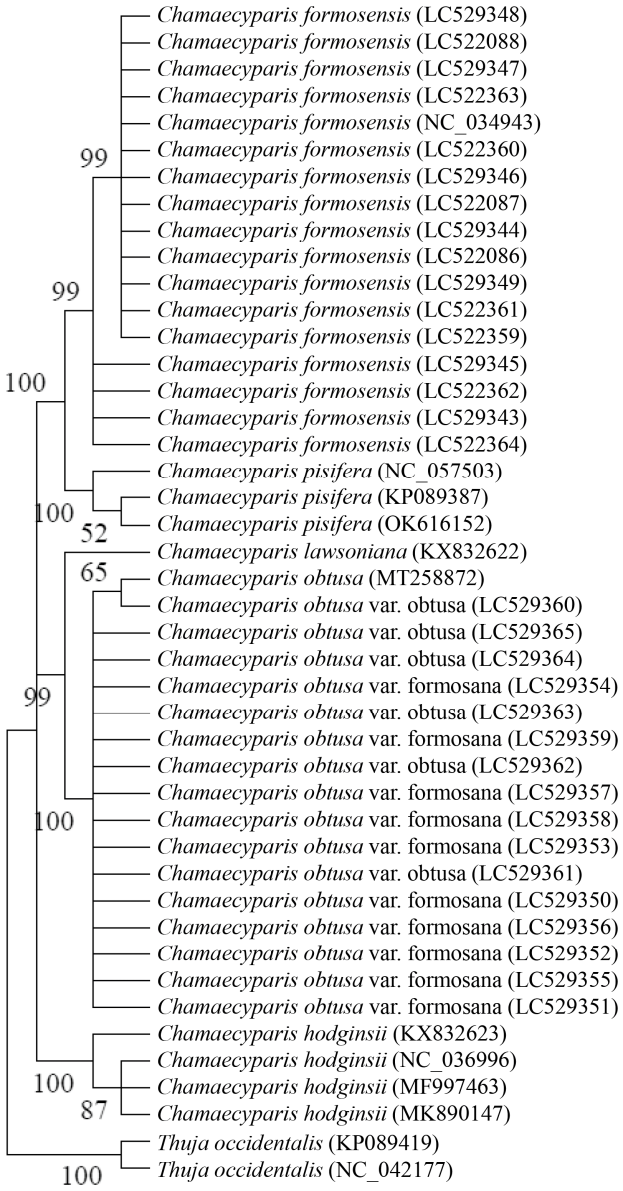

Figure 3. Phylogenetic analysis of five *Chamaecyparis* species (*C. formosensis*, *C. hodginsii*, *C. lawsoniana*, *C. obtusa*, and *C. pisifera*) using *ycf1*b. Phylogenetic analysis was performed with 1000 bootstraps using the Kimura two-parameter model in the MEGA (ver. 11.0) software using the maximum likelihood method.

3.4. Phylogenetic Analysis of Six Species of Chamaecyparis *Using* clpP

Since *clpP* gene sequences of *C. nootkatensis* have been published, we evaluated the functionality of *clpP* as a DNA barcode in six species (*C. formosensis*, *C. hodginsii*, *C. lawsoniana*, *C. nootkatensis*, *C. obtusa*, and *C. pisifera*) of *Chamaecyparis* (Figure 4). In addition to the nine reported complete chloroplast genes presented in Figure 1, 33 *clpP* sequences were analyzed. The length of the *clpP* for alignment was 540 bases, and the number of non-identical nucleotides was 45 bases (Supplementary Material Figure S3). The *clpP* nucleotide sequences of *C. formosensis* and *C. pisifera* were identical. In contrast, there was a mismatch of a single base among the 540-base alignment between *C. lawsoniana* and *C. obtusa*, making accurate species identification impossible. Previously, mutations in the 14th–33rd bases region of *clpP* have been shown to be important for the identification of

27 Actinidiaceae species [46]. In *Chamaecyparis* species, *clpP* had a mutation starting from the 103rd position, excluding *C. nootkatensis*. *clpP* has been proposed as a DNA barcode for 27 species of Actinidiaceae in a previous study; however, it cannot accurately identify all species [46].

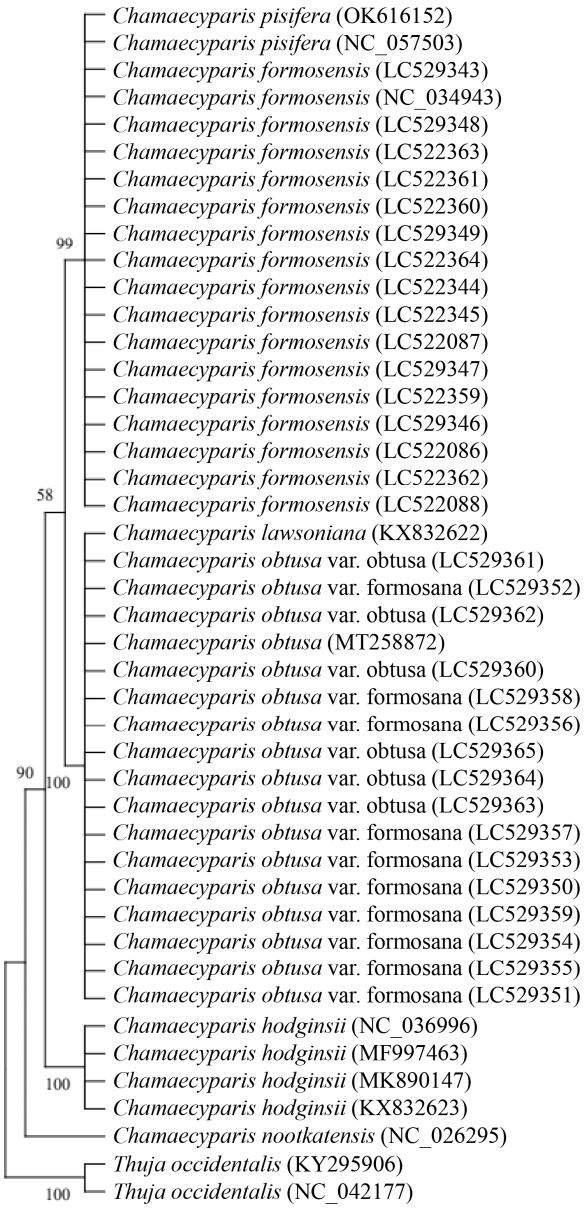

Figure 4. Phylogenetic analysis of six *Chamaecyparis* species (*C. formosensis*, *C. hodginsii*, *C. lawsoniana*, *C. nootkatensis*, *C. obtusa*, and *C. pisifera*) using *clpP*. Phylogenetic analysis was performed with 1000 bootstraps using the Kimura two-parameter model in the MEGA (ver. 11.0) software using the maximum likelihood method.

3.5. Phylogenetic Analysis of Six Species of Chamaecyparis *Using* accD

We investigated whether *accD* could be used as a DNA barcode for six species of *Chamaecyparis* (*C. formosensis*, *C. hodginsii*, *C. lawsoniana*, *C. nootkatensis*, *C. obtusa*, and *C. pisifera*) (Figure 5). In addition to the nine reported complete chloroplast genes presented in Figure 1, 33 *accD* sequences were analyzed. All six species formed an independent lineage. The nucleotide sequence of *accD* for alignment was 2208 bases, and the number of non-identical nucleotides was 299 bases (Supplementary Material Figure S4). This result was inconsistent with results presented in Figure 1, where *accD* showed the fourth-highest

nucleotide diversity; this was attributed to additional *accD* sequences for *C. nootkatensis*. The number of non-identical nucleotides, excluding those of *C. nootkatensis*, was 162 bases. *accD* could be used to distinguish between two subspecies, *C. obtusa* var. *obtusa* and *C. obtusa* var *formosana*. The *accD* sequence of *C. obtusa* had six mutations when compared to *accD* sequences of other species. Among them, three nucleotides (34th, 1098th, and 1544th) were identical across subspecies (Supplementary Material Figure S4). The *accD* gene had sufficient nucleotide diversity to identify six species. Amino acid repeats have been found in *accD*, and the number of repetitions differs depending on the species [55]. In *Chamaecyparis*, a region with repeats of 10 amino acid sequences was found at the beginning.

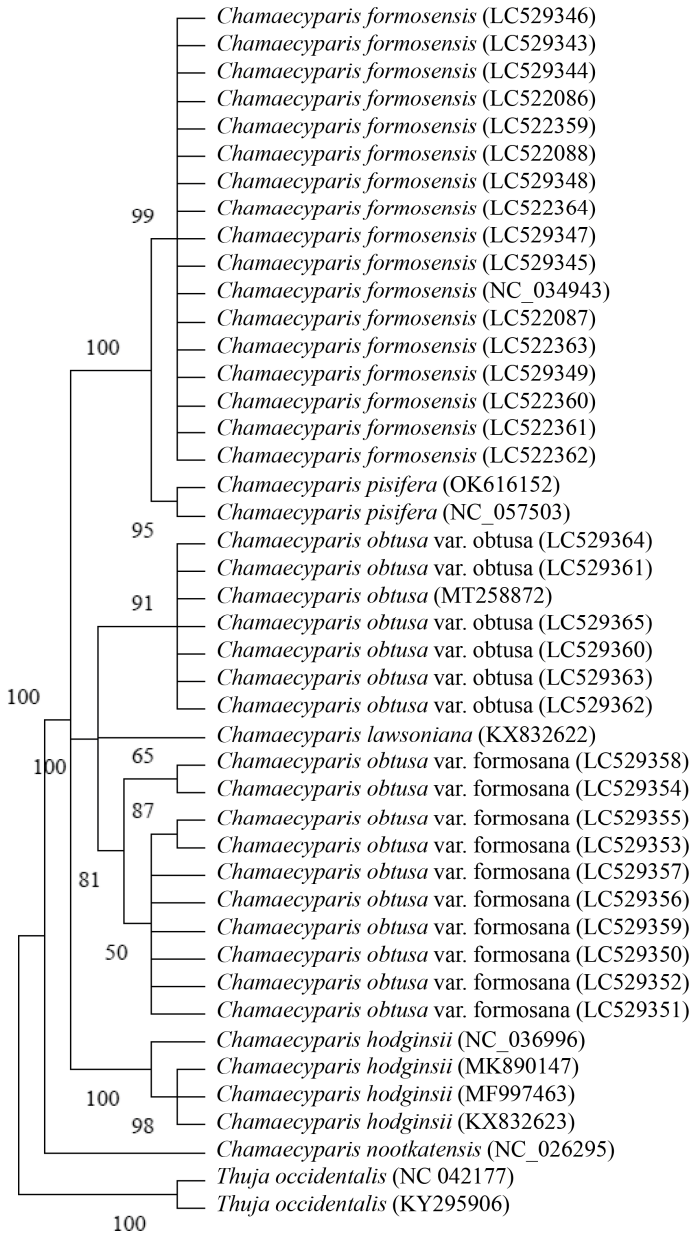

Figure 5. Phylogenetic analysis of six *Chamaecyparis* species (*C. formosensis*, *C. hodginsii*, *C. lawsoniana*, *C. nootkatensis*, *C. obtusa*, and *C. pisifera*) using *accD*. Phylogenetic analysis was performed with 1000 bootstraps using the Kimura two-parameter model in the MEGA (ver. 11.0) software using the maximum likelihood method.

3.6. Phylogenetic Analysis of Six Species of Chamaecyparis *Using* ycf2

ycf2 was evaluated as a DNA barcode for six species of *Chamaecyparis* (*C. formosensis*, *C. hodginsii*, *C. lawsoniana*, *C. nootkatensis*, *C. obtusa*, and *C. pisifera*) (Figure 6). In total,

42 reported *ycf2* sequences were analyzed. All six species could be identified, and nucleotide diversity was present within *C. formosensis*, forming six subspecies lineage groups. Unlike other genes, *ycf2* had variations in six nucleotides (3616th, 3629th, 3654th, 3655th, 3689th, and 3690th) within *C. formosensis* (Supplementary Material Figure S5). The nucleotide sequence of *ycf2* for alignment was 6748 bases, and the non-identical nucleotide was 720 bases (Supplementary Material Figure S5). Like *accD*, *ycf2* from *C. nootkatensis*, for which a complete genome sequence is unavailable, increased the nucleotide diversity compared to that presented in Figure 1. The non-identical nucleotide sequence, excluding *C. nootkatensis* sequences, was 396 bases. *ycf2* could be used to distinguish *C. obtusa* from other species as well as its subspecies.

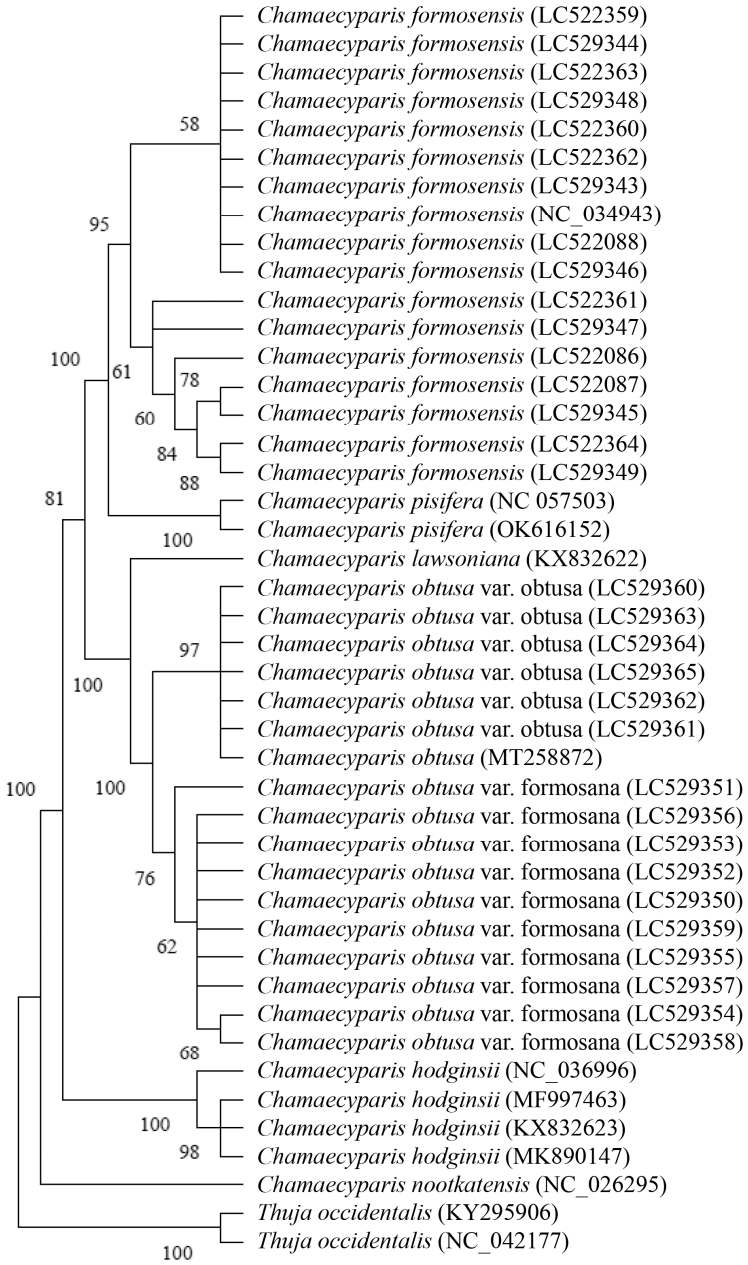

Figure 6. Phylogenetic analysis of five *Chamaecyparis* species (*C. formosensis*, *C. hodginsii*, *C. lawsoniana*, *C. nootkatensis*, *C. obtusa*, and *C. pisifera*) using *ycf2*. Phylogenetic analysis was performed with 1000 bootstraps using the Kimura two-parameter model in the MEGA (ver. 11.0) software using the maximum likelihood method.

3.7. *Phylogenetic Analysis of Five Species of* Chamaecyparis *Using* rps16

We investigated whether *rps16* could be used as a DNA barcode for five species of *Chamaecyparis* (*C. formosensis*, *C. hodginsii*, *C. lawsoniana*, *C. obtusa*, and *C. pisifera*) (Figure 7). In addition to the nine reported complete chloroplast genes presented in Figure 1, 41 *rps16* sequences were analyzed. The total nucleotide sequence of *rps16* was 374 bases, and the non-identical nucleotide was 24 bases (Supplementary Material Figure S6). *C. pisifera* and *C. formosensis* are phylogenetically similar, with a chloroplast DNA divergence of 0.57% [11]. *rps16* also had only one mutation at the 170th position in the alignment between *C. formosensis* and *C. pisifera*. Therefore, it could not be used to accurately identify the species.

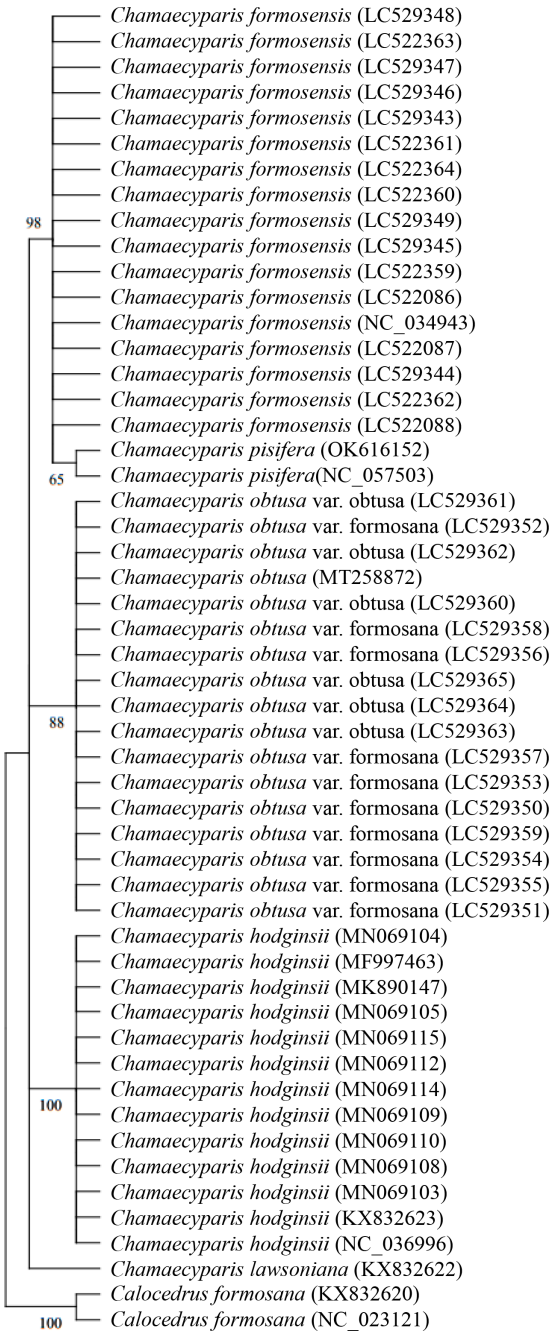

Figure 7. Phylogenetic analysis of five *Chamaecyparis* species (*C. formosensis*, *C. hodginsii*, *C. lawsoniana*, *C. obtusa*, and *C. pisifera*) using *rps16*. Phylogenetic analysis was performed with 1000 bootstraps using the Kimura two-parameter model in the MEGA (ver. 11.0) software using the maximum likelihood method.

4. Conclusions

In this study, we proposed three genes, *ycf1*b, *accD*, and *ycf2*, as DNA barcodes for the molecular and phylogenetic classification of the genus *Chamaecyparis*, which is difficult to distinguish by species based on anatomical characteristics alone. To select DNA barcodes for species identification, six candidate genes with high nucleotide diversity in chloroplast genes were selected, and the accuracy of species identification was evaluated via phylogenetic analysis. Based on all available *ycf1*b, *accD*, and *ycf2* sequences in the NCBI nucleotide database on 1 March 2024, these genes allowed the identification of *Chamaecyparis* species. In contrast, *trnP-GGG*, *clpP*, and *rps16* failed to accurately distinguish *C. pisifera* and *C. formosensis*. Therefore, using *ycf1*b, *accD*, and *ycf2* in phylogenetic analysis can accurately identify species of the genus *Chamaecyparis*. This study was analyzed based on genes reported to date, and new useful DNA barcodes may be proposed by the accumulation of additional genetic data; however, it can be used as an auxiliary method to clearly identify species in the genus *Chamaecyparis* when anatomical species identification is not possible. The method of exploring DNA barcodes in this study can be utilized as a method that can be used for taxonomic identification of subspecies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15071106/s1, Figure S1: Sequence alignment of 42 *trnP-GGG* sequences in the genus *Chamaecyparis*. The same base with the NC034943 represent as "." and the only different bases are presented. The species names of the sequences are presented in the last page; Figure S2: Sequence alignment of 42 *ycf1*b sequences in the genus *Chamaecyparis*. The same base with the KP089387 represent as "." and the only different bases are presented. The species names of the sequences are presented in the last page.; Figure S3: Sequence alignment of 42 *clpP* sequences in the genus *Chamaecyparis*. The same base with the KX832622 represent as "." and the only different bases are presented. The species names of the sequences are presented in the last page.; Figure S4: Sequence alignment of 42 *accD* sequences in the genus *Chamaecyparis*. The same base with the NC034943 represent as "." and the only different bases are presented. The species names of the sequences are presented in the last page.; Figure S5: Sequence alignment of 42 *ycf2* sequences in the genus *Chamaecyparis*. The same base with the LC522086 represent as "." and the only different bases are presented. The species names of the sequences are presented in the last page.; Figure S6: Sequence alignment of 50 *rps16* sequences in the genus *Chamaecyparis*. The same base with the OK616152 represent as "." and the only different bases are presented. The species names of the sequences are presented in the last page.

Author Contributions: Conceptualization, M.K. and T.-J.K.; methodology, M.K. and S.I.; software, S.I.; validation, M.K., S.I. and T.-J.K.; formal analysis, S.I. and T.-J.K.; investigation, S.I.; resources, T.-J.K.; data curation, S.I. and T.-J.K.; writing—original draft preparation, S.I.; writing—review and editing, T.-J.K.; visualization, S.I.; supervision, T.-J.K.; project administration, T.-J.K.; funding acquisition, M.K. and S.I. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets used/analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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