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Genetic Species Identification Using *ycf1*b, *rbcL*, and *trnH-psbA* in the Genus *Pinus* as a Complementary Method for Anatomical Wood Species Identification

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Abstract: This study proposes the use of genetic analysis as a complementary method for species identification in the genus *Pinus*, particularly in cases where anatomical identification is challenging. *Pinus* species were grouped based on anatomical similarities, and the efficacy of using *ycf1*b, which is the most variable for *Pinus* species identification, and *rbcL*, which is a suggested DNA barcode for land plants, was evaluated within each group. Sequences for each species were obtained from the National Center for Biotechnology Information database and were used to perform phylogenetic analysis. Among the species in Group 1 (*P. echinata*, *P. elliottii*, *P. ponderosa*, *P. radiata*, *P. rigida*, *P. taeda*, and *P. virginiana*), *rbcL* was only effective in identifying *P. radiata* and *P. ponderosa*, while *ycf1*b classified five species. An additional DNA barcode, *trnH-psbA*, was needed to identify *P. radiata* and *P. taeda*. In Group 2 (*P. densiflora*, *P. sylvestris*, and *P. thunbergii*), most species were identified using both *rbcL* and *ycf1*b, with the exception of possible hybrids of *P. densiflora* and *P. sylvestris*. In Group 3 (*P. koraiensis* and *P. strobus*), two species were identified using *rbcL* and *ycf1*b. Combining genetic species identification with anatomical identification can accurately identify species of the genus *Pinus*.

Keywords: genetic species identification; *trnH-psbA*; *Pinus*; wood; *ycf1*b

Citation: Kim, M.; Kim, T.-J. Genetic Species Identification Using *ycf1*b, *rbcL*, and *trnH-psbA* in the Genus *Pinus* as a Complementary Method for Anatomical Wood Species Identification. *Forests* **2023**, *14*, 1095. https://doi.org/10.3390/f14061095

Academic Editors: Carmen-Mihaela Popescu, Jalel Labidi and Darci Alberto Gatto

Received: 5 April 2023
Revised: 10 May 2023
Accepted: 24 May 2023
Published: 25 May 2023

1. Introduction

Accurately identifying the species of wood is very important in many tasks, such as forensic identification of illegal timber, restoration without damaging the value of cultural assets, and use of industrially appropriate species [1]. Wood species were mainly identified through anatomy analysis using a microscope [2–6]. However, it may sometimes be difficult to distinguish between structurally similar species based on anatomical features alone. DNA barcoding can provide an alternative and complementary means to identify species of wood by analyzing the gene sequences [7]. It is difficult to obtain DNA for genetic analysis from wood that has been processed, including drying, and has been in use for a prolonged period. However, a method for successfully extracting DNA from old wood using sandpaper and an improved extraction method has been proposed [8].

In addition to DNA barcoding, fluorescence in situ hybridization (FISH) methods are another approach that can be used to identify wood species [9]. FISH utilizes short DNA or RNA probes that selectively bind to a target sequence of interest in a complementary manner. These probes are labeled with fluorescent molecules, which enable the visualization of the presence and location of the target nucleic acid sequences in the wood samples. Target nucleic acid sequences for the FISH method for wood species identification include telomere repeat, centromeric repeat, and rRNA [10]. However, one of the major challenges in using the FISH method to identify plant species is the development of appropriate probes for each species [11].

In addition to FISH, other methods such as random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphisms (RFLP) have been used for plant

species identification [12]. While conventional PCR selectively amplifies only the target gene, RAPD performs non-specific gene amplification using short random primers to compare different-sized fragment patterns for species identification. However, a potential weakness with RAPD is the low reproducibility of different-sized fragment patterns obtained in each experiment [12]. RFLP is another method that can suggest a change in the nucleotide sequence of a specific site by observing the pattern of the cut fragment due to the difference in the nucleotide sequence of the DNA site cut by the restriction enzyme. However, a library of polymorphisms of each species is required for plant species identification using RFLP, and the average probability of distinguishing two alleles taken at random is not high [12]. Therefore, in this study, we propose a method for wood species identification using phylogenetic analysis based on the entire nucleotide sequence of DNA barcodes that have been shown to be useful in plant species identification. Although this method may not be suitable for identifying species of the entire *Pinus* genus, it can be used effectively to identify a limited number of species for which anatomical species identification in wood is difficult.

The genus *Pinus* has an important ecological role in providing habitats for wildlife. Furthermore, its extracts exhibit antimicrobial, antidiabetic, and anti-inflammatory activities [13–16]. It accounted for approximately 36% of Korea's medium-density fiberboard raw materials in 2008 [17]. In addition, the genus *Pinus* occupied more than 22% of Korean forests in 2015 [18]. In addition, *Pinus* was used as building material for architectural heritage sites in Korea [19]. Therefore, it is important to accurately identify the wood species of the genus *Pinus*. Among the species of the genus *Pinus*, species that are anatomically difficult to distinguish from each other are classified into three groups in Table 1. Species in Group 1 share anatomical features such as distinct growth-ring boundaries, pinoids, and thin-walled epithelial cells [20–27]. Species in Group 2 are characterized by window-like cross-field pits and thin-walled epithelial cells [20,26,28]. Species in Group 3 are characterized by gradual transition from early wood to late wood, thin-walled epithelial cells, and window-like cross-field pits [20]. These common structural features make anatomical identification difficult through microscopic observation.

Plant cells have a characteristic organelle, the plastid, and the plastid has a genome independent of the nucleus and mitochondria. It has been proposed that plant species can be identified using plastid genomes owing to several advantages, such as high copy number, conserved regions, variable regions, rapid evolution, and maternal inheritance [2,3]. In addition, the plastid genome has a higher number of reported nucleotide sequences compared to mitochondrial or nuclear genomes in plants, thus facilitating DNA barcode analysis for species identification [29,30]. In previous studies, *matK*, *rbcL*, *rpoB*, and *ycf1* were suggested as genes for DNA barcoding [31–33]. *matK* and *rbcL* were suggested as DNA barcodes for land plants by the Consortium for the Barcode of Life—Plant Working Group [34]. *rpoB* was suggested to be more efficient in family level identification than genus level identification [35]. Although *ycf1* has been proposed as the most promising plastid DNA barcode for terrestrial plants [30], it is a very large gene encoding approximately 1800 amino acids. As part of the coding region of *ycf1*, *ycf1*b with a size of about 1 kb was suggested to be more efficient in species identification in a previous study [36].

In the previous study, effectiveness of the DNA barcoding for species identification in the genus *Pinus* using *matK*, *rbcL*, *trnH-psbA*, *trnL-trnF*, *rpl20-rps18*, *trnV*, *ycf1*, and *ycf2* was evaluated [37]. However, the results did not provide clear evidence for the possibility to identify genetically closely related species, such as *P. mugo*, *P. uncinata*, and *P. uliginosa* [37]. In this study, we evaluated whether *rbcL* and *ycf1*b could be used for genetic species identification within small groups of the genus *Pinus*, where species identification was difficult by anatomical observation, based on available genetic information reported to date. When the species could not be identified by *ycf1*b, the possibility of species identification by *trnH-psbA* was additionally evaluated by limiting the species to which species could not be identified. *rbcL* and *ycf1*b were evaluated in this study because *rbcL* is a suggested DNA barcode for land plants, and *ycf1* is the most variable in *Pinus* species identification [37].

matK was not used in this study because the probability of species identification of the genus *Pinus* was low, i.e., 23% [30].

Table 1. Species groups of the genus *Pinus* that are difficult-to-identify species owing to similar anatomical characteristics under a microscope.

Group	Gene	Species Name	NCBI Accession Number
Group 1	*rbcL*	*P. echinata*	AY724754, AY947435, JN854204, MZ424449, NC_065458
		P. elliottii	AY724755, JN854202, NC_042788
		P. ponderosa	AY497234, DQ353721, FJ899555, JN854171, JN854172, NC_067715
		P. radiata	AY497250, JN854165, X58134
		P. rigida	AY724757, JN854163, JQ512587, JQ512589, MZ424450, NC_065459,
		P. taeda	AF119177, AY724758, FJ899561, JQ512592, KC427273, KY964286
		P. virginiana	AY947430, JN854155, JQ512596
	*ycf1*b	*P. echinata*	KC157080, KC157180, JN854204, MZ424449, NC_065458
		P. elliottii	JN854202, KC157104, NC_042788
		P. ponderosa	FJ899555, JN854171, JN854172, KC157087, KC157140, KC157195, KP089392, KP128671
		P. radiata	JN854165, KC157129,
		P. rigida	JN854163, KC157079, KC157177, KP128673, KP205539, MZ424450, NC_065459, NC_067715, OL547484
		P. taeda	FJ899561, KC157082, KC427273, KY964286
		P. virginiana	JN854155, KC157196
	trnH-psbA	*P. radiata*	FR832544, JN854165, KC157276, KC157332, KC157399,
		P. taeda	FJ899561, KC157213, KC427273, KY964286, MF945991, MK895630
Group 2	*rbcL*	*P. densiflora*	JN854210, MF990371, MT786135, MZ677091, NC_042394, NC_062639, NC_062640
		P. sylvestris	JN854158, KR476379, MT787466, MT796488
		P. thunbergii	D17510, JQ512594, MH612862, MW599991
	*ycf1*b	*P. densiflora*	JN854210, KP089385, MF990371, MT786135, MZ677091, NC_042394, NC_062639, NC_062640
		P. sylvestris	JN854158, KP089414, KR476379, MT787466, MT796488
		P. thunbergii	D17510, FJ899562, KP089381, MH612862, MW599991
Group 3	*rbcL*	*P. koraiensis*	AB019797, AY228468, EF440596, JQ512578, JQ512579, NC_004677
		P. strobus	AB019798, AF479880, AY497219, FJ899560, JQ512590, KP099650, NC_026302
	*ycf1*b	*P. koraiensis*	AY228468, KP089410, KP128638, KP128639, NC_004677
		P. strobus	FJ899560, KP089389, KP099650, KP128655, KP128656, NC_026302

2. Research Methods and Data Sources

2.1. Sequences of rbcL, ycf1b, and trnH-psbA in the Genus Pinus

Gene sequences covering more than 90% of the *rbcL*, *ycf1*b, and *trnH-psbA* sequences from the species of the genus *Pinus* were collected from the National Center for Biotechnology Information (NCBI) gene database (Table 1). In the case of *trnH-psbA*, only the sequences of *P. taeda* and *P. radiata*, which could not be distinguished by *ycf1*b, were analyzed. For sequences deposited with the chloroplast genome or whole plastid, the nucleotide sequence of the corresponding gene was obtained through alignment using known base sequences of *rbcL*, *ycf1*b, and *trnH-psbA* from the same species [38].

2.2. Gene Alignment and Phylogenetic Analysis

Nucleotide sequence alignment of the collected genes was performed using ClustalW [38]. The aligned sequences were exported using BioEdit 7.2. [39]. In this study, the size of the compared sequence was presented for each group using aligned sequences. The ability of each gene to identify species was assessed by the number of non-identical sites. The phylogenetic analysis was conducted using the maximum likelihood method and the Kimura 2-parameter model with 1000 bootstrap replications in the MEGA 11 software [40]. A cut-off value of 50% was applied to the condensed tree. As an outgroup, the corresponding genes of *Chamaecyparis pisifera*, which belongs to the same class, Pinopsida, as the genus *Pinus*, were used.

3. Results and Discussion

3.1. Species Identification of *P. echinata*, *P. elliottii*, *P. ponderosa*, *P. radiata*, *P. rigida*, *P. taeda*, and *P. virginiana* in Group 1 of Table 1 through Phylogenetic Analysis

Phylogenetic analysis was performed using the *rbcL* and *ycf1*b gene sequences in Group 1 of Table 1, where it is difficult to identify species by anatomical microscopic observation (Figures 1 and 2, respectively). When using *rbcL*, only two species, *P. radiata* and *P. ponderosa*, were classified independently from other species, so *rbcL* could be used for identification of these two species. However, it was difficult to distinguish between *P. echinata* and *P. elliottii*, between *P. taeda* and *P. virginiana*, and between *P. rigida* and *P. taeda* (Figure 1). In the phylogenetic analysis using *ycf1*b (Figure 2), five species were classified independently and the remaining two species, *P. taeda* and *P. radiata*, could not be distinguished. In the genus *Pinus*, *rbcL*b showed discrimination success of more than 20%, and *ycf1*b showed discrimination success of more than 50% [30]. This is consistent with the higher discrimination success of ycf1b in our results.

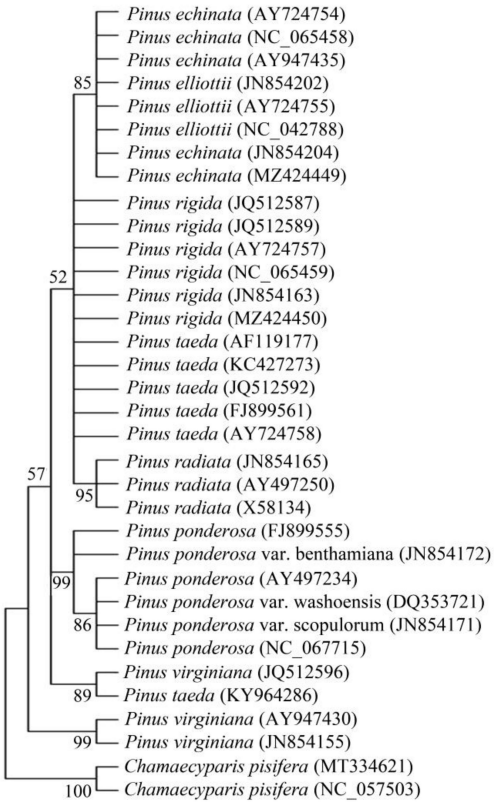

Figure 1. Species identification of *P. echinata*, *P. elliottii*, *P. ponderosa*, *P. radiata*, *P. rigida*, *P. taeda*, and *P. virginiana* in Group 1 of Table 1 through phylogenetic analysis using *rbcL* gene sequences.

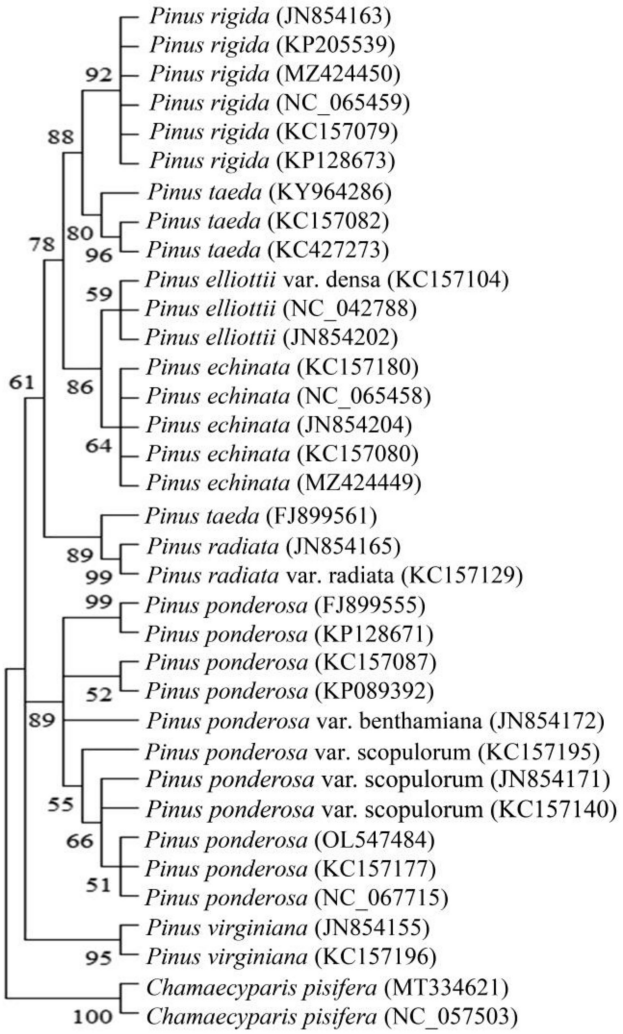

Figure 2. Species identification of *P. echinata*, *P. elliottii*, *P. ponderosa*, *P. radiata*, *P. rigida*, *P. taeda*, and *P. virginiana* in Group 1 of Table 1 through phylogenetic analysis using *ycf1*b gene sequences.

In general, single nucleotide polymorphism genotyping is one of the effective tools in DNA barcoding [41,42], but while it is suitable for identification of individuals, it has limitations in distinguishing species [8]. The size of the compared sequences used for species identification and the ratio of bases that show variable sites between species or individuals are summarized in Table 2. As the overhang parts were trimmed after alignment, the size of the compared sequences used in this study is slightly different compared to other studies. In the comparison of *rbcL* in Group 1, 2.4% of the bases were non-identical. In the case of *ycf1*b in Group 1, the size of the compared base sequence was 1057 bases, which was smaller than the 1325 bases of the compared region of *rbcL*, but the number of non-identical bases was 83, which was more than 32 of *rbcL*. This different non-identical base ratio suggested that *ycf1*b had more information for species identification than *rbcL*. In the results of species identification, five species were independently classified by *ycf1*b compared to two species independently classified by *rbcL* in Group 1. Therefore, *ycf1*b is more useful for species identification than *rbcL* in Group 1.

Table 2. Sequence information used for genetic species identification in three groups.

Group	Gene Name	Compared Sequence Size (Bases)	Number of Non-Identical Sites (Percentage of Non-Identical Bases)
1	*rbcL*	1325	32 (2.4%)
	*ycf1*b	1057	83 (7.9%)
2	*rbcL*	1427	18 (1.3%)
	*ycf1*b	1184	27 (2.3%)
3	*rbcL*	1302	7 (0.5%)
	*ycf1*b	1256	22 (1.8%)

According to a study on DNA barcoding of genera other than *Pinus*, the discrimination success percentage of *ycf1*b was found to be about 60% in Iris, 100% in Armeniaca, 50% in Paeonia, 40% in Quercus, and approximately 60% in Panax [30]. Additionally, the discrimination success percentage of *rbcL*b, a part of *rbcL*, was about 50%, 0%, 10%, 25%, and 60%, respectively [30]. These results suggest that *ycf1*b is more effective in discriminating between closely related species compared to *rbcL*b, which has a lower discrimination success percentage. Other previous studies have also reported that *rbcL* is unsuitable for identifying species of *Sweretia chirayita* and its adulterant species, as well as species of the genus *Ardisia* [43,44]. These previous results in many plants support that *ycf1*b is better than *rbcL* in species identification.

To discriminate *P. radiata* and *P. taeda*, which are indistinguishable even in species identification using *ycf1*b, species identification was performed using *trnH-psbA*, which was proposed to be used for tree species identification (Figure 3). The non-coding spacer region, *trnH-psbA*, has been extensively utilized for DNA barcoding in the past [45–47]. However, due to its lower primer universality compared to *rbcL* and difficulties in alignments caused by its variable sequence size [48,49], *trnH-psbA* was not used as the primary analysis DNA region in this study. Phylogenetic analysis using *trnH-psbA* classified *P. radiata* and *P. taeda* independently. Therefore, according to the currently available genetic information, if *ycf1*b is used for species identification in Group 1 and *trnH-psbA* is used additionally, the identification of seven species, which are anatomically difficult-to-identify species, is possible by genetic analysis. This result is consistent with a previous study showing a higher discrimination success percentage of *trnH-psbA* than *rbcL*b but lower than *ycf1*b in the genus *Pinus* [30]. In order to show in detail which bases differ between species, the nucleotide sequences of species that are difficult to classify in Figures 1 and 2 are aligned (Supplementary Figures S1–S4).

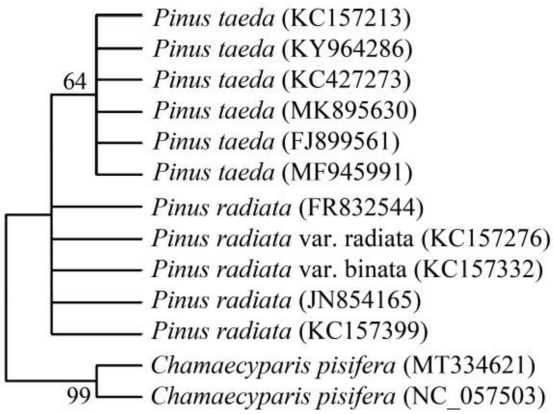

Figure 3. Species identification of *P. radiata* and *P. taeda* through phylogenetic analysis using *trnH-psbA* sequences.

3.2. Species Identification of P. densiflora, P. sylvestris, and P. thunbergii in Group 2 of Table 1 through Phylogenetic Analysis

In Group 2, both genes, *rbcL* and *ycf1*b, were clearly identified in *P. thunbergii*, but could not differentiate between *P. densiflora* and *P. sylvestris* (Figure 4). In a previous study, *ycf1* also did not distinguish well between *P. densiflora* and *P. sylvestris* [33]. In China, the possibility of hybridization between *P. densiflora* and *P. sylvestris* has been reported because of overlapping habitats [35,50]. The *P. densiflora* genes used in this study, MZ677091, NC_062639, and NC_062640, are plastid genes of species from China. If such hybridization has happened, species identification using the chloroplast gene will be fundamentally impossible between *P. densiflora* and *P. sylvestris*. In order to show the difference in nucleotide sequence between *P. densiflora* and *P. sylvestris*, which are difficult to discriminate in Figure 4, the nucleotide sequences of *rbcL* and *ycf1*b from the species were aligned (Supplementary Figures S5 and S6). Many bases of *P. densiflora*, MZ677091, NC_062639, and NC_062640, were identical to the corresponding bases of *P. sylvestris*. This observation supported the hybridization between *P. densiflora* and *P. sylvestris* in China. Excluding individuals presumed to have hybridization, phylogenetic analysis using *rbcL* and *ycf1*b can discriminate the species of *P. densiflora* and *P. sylvestris*. In Group 2, the ratio of non-identical bases in *rbcL* and *ycf1*b was 1.3% and 2.3%, respectively, and there was no significant difference (Table 2).

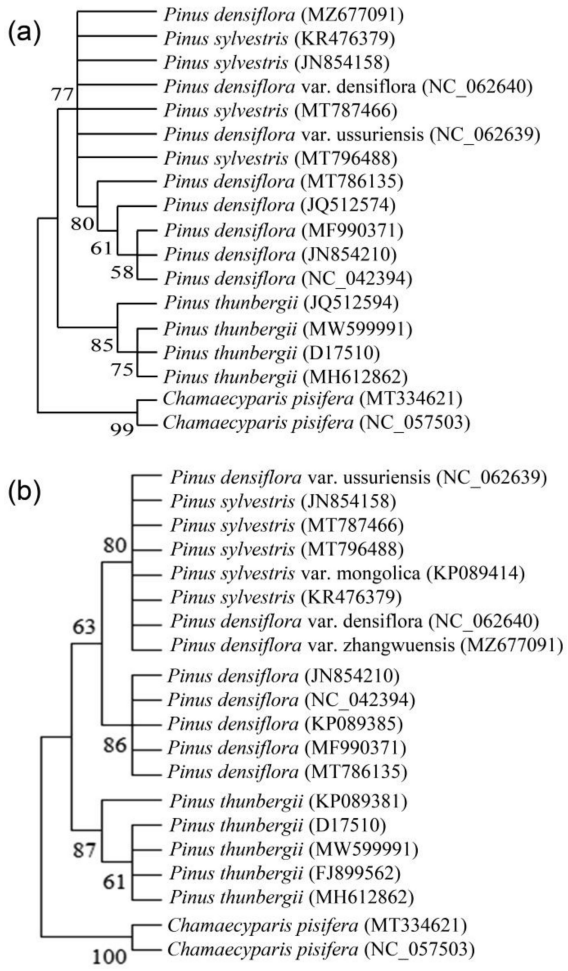

Figure 4. Species identification of *P. densiflora*, *P. sylvestris*, and *P. thunbergii* in Group 2 of Table 1 through phylogenetic analysis using (**a**) *rbcL* and (**b**) *ycf1*b gene sequences.

In addition to species identification using DNA barcoding, there was also a study that identified *P. densiflora* and *P. sylvestris* through a chemical analysis method using near-

infrared spectroscopy and multivariate analysis [19]. *P. densiflora* and *P. thunbergii* were also identified through the same method [51]. However, since the results were not obtained for *P. densiflora* and *P. sylvestris* in China, additional research is needed to understand the differences in the case of hybridized species. These previous studies suggest the possibility of using chemical methods for identifying wood between anatomically similar species, in addition to biological methods.

3.3. Species Identification of P. koraiensis and P. strobus in Group 3 of Table 1 through Phylogenetic Analysis

In Group 3, *P. koraiensis* and *P. strobus* were each independently classified in phylogenetic analysis using *rbcL* and *ycf1*b (Figure 5). The ratio of non-identical bases in *rbcL* and *ycf1*b was 0.5% and 1.8%, respectively, which were low compared to other groups (Table 2). Despite these low base variations, they showed high species–specific discrimination and contained sufficient information for species identification of *P. koraiensis* and *P. strobus*.

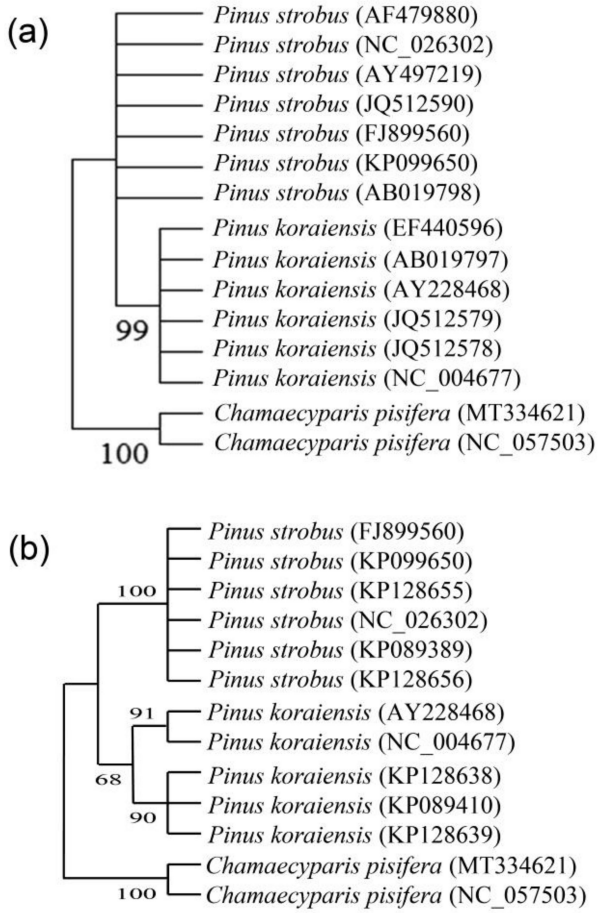

Figure 5. Species identification of *P. koraiensis* and *P. strobus* in Group 3 of Table 1 through phylogenetic analysis using (**a**) *rbcL* and (**b**) *ycf1*b gene sequences.

The present study indicates that *ycf1*b is a more suitable DNA barcode for species identification in all three species groups of the genus *Pinus* in Table 1 compared to *rbcL*, as *ycf1*b was more variable. This finding is consistent with a previous study that observed a non-identical base ratio of approximately 22% in *ycf1* from 55 *Pinus* species [33]. Furthermore, previous study has also indicated that *rbcL* is more effective in identifying species in lower plants compared to seed plants [52]. Notably, our results demonstrated the efficacy of *ycf1*b in discriminating between species in the genus *Pinus*, which are challenging to identify anatomically. However, it is worth noting that the use of *ycf1*b as DNA barcode for species identification may not be universally applicable to all plants, given that the family

Poaceae has a severe deletion of the *ycf1* gene [53]. Therefore, it is essential to confirm the presence or absence of such a gene deletion when using *ycf1* for identifying plant species other than the genus *Pinus*.

Despite the use of various DNA barcodes, accurately identifying all species within the genus *Pinus* remains a challenge [30,37]. This underscores the limitations of relying solely on DNA barcodes for species identification. Therefore, for accurate identification of wood species within the genus *Pinus*, anatomical identification can be used first, followed by molecular identification using *ycf1*b and *trnH-psbA* as DNA barcodes.

4. Conclusions

In the genus *Pinus*, while identifying species by anatomical analysis of wood is difficult, genetic analysis can be used as a supplementary method to identify species accurately. Among a group comprising seven species, i.e., *P. echinata*, *P. elliottii*, *P. ponderosa*, *P. radiata*, *P. rigida*, *P. taeda*, and *P. virginiana*, five species can be identified using *ycf1*b, while *P. radiata* and *P. taeda* could be identified using *trnH-psbA* in addition. In Group 2 with three species, i.e., *P. densiflora*, *P. sylvestris*, and *P. thunbergii*, *P. thunbergii* could be identified using *rbcL* and *ycf1*b, and *P. densiflora* and *P. sylvestris* could also be classified, except for individuals in which hybridization may have occurred. In Group 3 with *P. koraiensis* and *P. strobus*, two species were independently classified using *rbcL* and *ycf1*b. This study suggests that wood species of the genus *Pinus* can be first identified anatomically using microscopic methods. Additionally, the DNA barcoding method using *ycf1*b and *trnH-psbA* can be used to identify species that are difficult to identify anatomically, in order to accurately identify the wood species of the genus *Pinus*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14061095/s1, Figure S1: Alignment of *rbcL* from *P. echinata* and *P. elliottii*; Figure S2: Alignment of *rbcL* from *P. rigida* and *P. taeda*; Figure S3: Alignment of *rbcL* from *P. taeda* and *P. virginiana*; Figure S4: Alignment of *ycf1*b from *P. radiata* and *P. taeda*; Figure S5: Alignment of *rbcL* from *P. densiflora* and *P. sylvestris*; Figure S6: Alignment of *ycf1*b from *P. densiflora* and *P. sylvestris*.

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

Funding: This paper was supported by the Korea Institute for Advancement of Technology (KIAT) grant funded by the Ministry of Trade, Industry and Energy (MOTIE) (P0012725).

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to intellectual property protection.

Acknowledgments: All authors thank Jung-Ae Oh from the Korea Forestry Promotion Institute for suggesting three groups that are difficult-to-identify species of the genus *Pinus* owing to their similar anatomical structures.

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

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