Kusumoto *et al. Journal of Wood Science* (2022) 68:58
https://doi.org/10.1186/s10086-022-02066-x

Journal of Wood Science

ORIGINAL ARTICLE

Open Access

The variability of terpenoids and flavonoids in native *Lindera umbellata* from the same region

Norihisa Kusumoto*, Takuya Morikawa, Koh Hashida, Naoyuki Matsui and Tatsuro Ohira

Abstract

The leaves and twigs of *Lindera* spp. have long been used as a herbal medicine and toothpicks in Japan. However, little is known about individual variations in the extractives of these species, because many previous studies have not distinguished extractives between individuals. In this study, we investigated the extractives of *L. umbellata* at the individual level. The detailed identification of the inter- and intra-individual variations in the major terpenoids and flavonoids in native *L. umbellata* may greatly contribute to the development of cultivation techniques and the effective use of forest resources. The contents of major components of *L. umbellata*, including four terpenoids (1,8-cineole, linalool, geraniol, and geranyl acetate) and five low-molecular-weight phenolics (pinocembrin chalcone, pinocembrin, pinostrobin chalcone, pinostrobin, and 5,6-dehydrokawain), were analyzed in leaves and twigs seasonally (June, August, and October). The compositions of the major terpenoids were strongly dependent on the properties of each individual and were generally independent of leaves and twigs. Moreover, geranyl acetate was characteristically present in the twigs of some individuals. As new findings regarding linalool, some individuals showed characteristic enantiomeric excesses, presumably because of biotic factors, and the proportion of these enantiomers was kept constant in each individual, regardless of the season. The total phenolic contents in leaves were more than twice those detected in twigs, and the leaves tended to contain more chalcones and twigs more flavanones. Furthermore, the contents of chalcones (pinocembrin chalcone vs. pinostrobin chalcone) and flavanones (pinocembrin vs. pinostrobin) were positively correlated in both leaves and twigs. The coefficient of variation (CV) clearly showed that the content of the major terpenoids was determined by inter-individual rather than intra-individual differences. Although the results obtained in this study should at present only be applicable to a limited population native to specific regions, our findings provide key knowledge in considering the sustainable use of *L. umbellata*.

Keywords: *Lindera umbellata*, Individual variation, Leaves, Twigs, Terpenoids, Flavonoids

Introduction

There are more than 100 species of the genus *Lindera* (*Lauraceae*) worldwide, 7 of which are native to Japan [1]. The leaves and twigs of these *Lindera* spp. have long been used as a herbal medicine called "Usho" [2] and toothpicks, as they are fragrant and have sedative and anti-inflammatory effects [3]. The essential oils of *Lindera* spp. were widely used as a perfume for soap in Japan before World War II [4]; however, their industrial use has since declined. In recent years, the value of native *Lindera* spp. as a unique Japanese herb has been re-recognized, and scientific evidence, such as the subjective and physiological effects of their tea [5] and the relaxation effect of their essential oils [6], have led to active approaches toward their expanded use in Japan.

Currently, the genus *Lindera* includes three species, with the genus being generally referred to as "Kuromoji" in Japan (Kuromoji: *L. umbellata*, Ke-kuromoji:

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L. sericea; and Hime-kuromoji: *L. lancea*). The main variations include Oba-Kuromoji (*L. umbellata* var. *membranacea*) and Usuge-kuromoji (*L. sericea* var. *glabrata*), among others [7, 8]. In general, the extractive components contained in these trees often greatly vary depending on the species and tissue of the tree, and significant differences have been reported even within the genus *Lindera*, which grows naturally in Japan. Hayashi and Komae [4, 9, 10] analyzed the composition of terpenoids in the essential oils extracted from the leaves of the five *Lindera* species mentioned above and reported that *L. umbellata* and *L. umbellata* var. *membranacea* have a very similar composition, with *L. sericea* containing less linalool and *L. lancea* containing more carvone. In the same research, phylogenetic classification was also discussed from a chemotaxonomic point of view, but no clear chemical classification was reached because of the difficulty of classification solely based on morphology and the presence of intermediate compositions. Conversely, although little is known about the interspecific variation in alkaloids, flavonoids, and other compounds in the genus *Lindera*, many reports have addressed the pharmacologically active components, which are well reviewed by Cao et al. [11]. In recent years, novel pharmacologically active constituents with unique chemical structures have been reported, such as linderapyrone, a monoterpene hydrocarbon bound to 5,6-dehydrokawain (a type of kavalactone) [12].

Little is known about the inter-individual (i.e., differences in extractives caused by genetic background, etc.) and intra-individual variations (i.e., differences in extractives attributed to tissues or seasons) in the genus *Lindera*. This is because many previous studies have not differentiated extractives among individuals, much less between leaves and twigs, e.g., in studies of essential oils. Recently, the discovery that the enantiomer ratio of linalool, which is the major oxygenated monoterpenoid in the leaves of *L. umbellata* var. *membranacea*, tends to favor the (*R*)-(−) form in leaves and in the (*S*)-(+) form in twigs, was reported by Inoue et al. [13]. Moreover, the four types of linalool oxide (furanoid) have been identified in their leaves and twigs according to conformational predominance [14]. Although these results were not examined in a single individual, they are important findings indicative of intra-individual variation in linalool. Moreover, it is well-known that the composition of terpenoids differs between the leaves and twigs of *L. umbellata*, and it has been proposed that there may be seasonal differences in the yield and content of each of its essential oil [2]. Nevertheless, these studies have all focused on populations located in specific regions, and there are no examples of detailed studies of inter-individual similarities or seasonal variations based on the results of chemical analyses in single individuals.

Therefore, this study aimed to investigate the resource properties of the extractives of *L. umbellata* and to assess the differences in the composition and content of major terpenoids and phenols of its native populations at the individual level in the same region. The clarification of the variation in the chemical composition and content of *L. umbellata* within the same region, which is often considered equal as a raw material, will greatly contribute to the development of cultivation techniques and the effective use of forest resources by utilizing the chemical properties of these trees.

Experimental

Plant materials

The sample collection region was located at the northern end of the Boso Hills, in the central part of the Boso Peninsula in Chiba Prefecture, Japan. The climate of this region is classified as warm-temperate. The native *L. umbellata* individuals were selected from three sites (Plot A: Okubo, Ichihara City; Plot B: Tozaki, Kimitsu City; and Plot C: Kanosan, Kimitsu City), as shown in Fig. 1. The leaves and twigs were collected from each individual (A1–A5, B1–B5, and C1–C5) in June, August, and October 2019, respectively. The altitude of the sample collection plots A, B, and C were 239, 164, and 293 m, respectively. In October, because defoliation was advanced in three individuals (i.e., A2, A3, and A5), it was not possible to ensure a sufficient amount of green leaves at the time of sample collection. The distance between individuals at each site was about 5 m. Twigs (diameter, < 5 mm) with healthy leaves were collected from the tip part of a tree at each collection timepoint. After the sample collection, the leaves and twigs were separated at

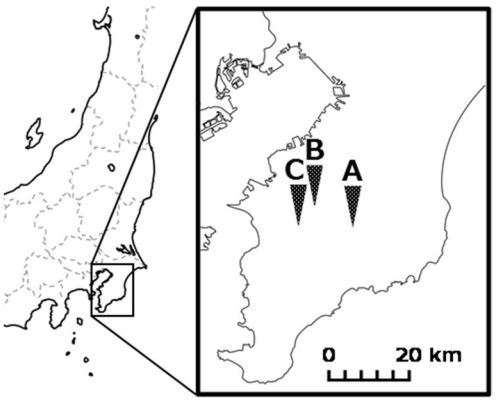

Fig. 1 Collection sites of the *Lindera umbellata* samples. Plots A (Okubo, Ichihara City), B (Tozaki, Kimitsu City), and C (Kanosan, Kimitsu City) indicate the plotting locations

the joint of the petiole (Fig. 2) and cut with scissors to about 1 cm each. They were then coarsely ground using a crash mill (MX-1100XTM, WARING COMMERCIAL, Stamford, CT, USA) in fresh conditions. Part of the crushed sample (ca. 0.5 g) was dried at 105 °C for 48 h, and the water content was measured based on the weight change. The average water content was 67.6% and 48.2% in leaves and twigs, respectively. The remainder of the crushed material was frozen at − 30 °C until extraction.

Chemicals

The monoterpenes (−)-linalool and geranyl acetate were obtained from Sigma–Aldrich (Tokyo, Japan); 1,8-cineole, geraniol, and pentadecane from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); (+)-catechin from Funakoshi Co., Ltd. (Tokyo, Japan); and dimethyl sulfone from FUJIFILM Wako Pure Chemical Co., Ltd. (Tokyo, Japan).

Quantification of monoterpenes

One gram of each milled sample was steeped in 10 mL of *n*-hexane containing 0.05 mg/mL of pentadecane, as an internal standard (IS), and was kept at room temperature (ca. 25 °C) for 24 h. One microliter of the supernatant from the *n*-hexane extract was injected into a gas chromatography–mass spectrometry (GC–MS) system (GCMS-QP2010 Ultra; Shimadzu Co., Ltd., Kyoto, Japan) equipped with a DB-5 ms UltraInert capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies Ltd., CA, USA). The temperature program was as follows: hold at 40 °C for 3 min, increase from 40 to 280 °C at a rate of 4 °C/min with final isothermal hold at 280 °C for 7 min. Helium was used as the carrier gas at a flow rate of 2 mL/min with a split ratio of 1:20, the injector temperature was 200 °C, and the detector temperature was 220 °C. Mass spectra were recorded over a 40–300 amu range at 3.3 scans/s with an ionization energy of 70 eV. Each component was identified by comparing its mass spectrum with a mass spectral library (NIST14, Wiley12 and FFNSC3) and with the spectra available in the literature [15].

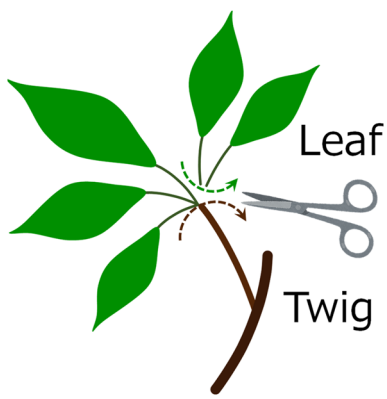

Fig. 2 Illustration of the leaf and twig separation point of the samples

Five-point calibration curves were constructed prior to the analysis of the samples based on the ratios obtained between the peak area of the IS (pentadecane) and those of four authentic standards, (−)-linalool (98.3%), 1,8-cineole (99.9%), geraniol (99.8%), and geranyl acetate (99.1%), which were analyzed using the GC–MS conditions mentioned above. The purity (%) of each authentic standard was measured based on the peaks of the GC–MS total ion current chromatogram. The contents of four major compounds, linalool, 1,8-cineole, geraniol, and geranyl acetate, were calculated based on the external standard method by substituting the peak area ratio of pentadecane and the constituent for each calibration curve. The calculated amounts of the four constituents were converted into the dry weight (dw) percentage of the actual sample weight (100%). The precision of terpene measurements in stored samples was deduced to be approximately 10% based on the replicate analyses of a powdered twigs.

Enantiomeric analysis of linalool

One microliter of the supernatant from the *n*-hexane extract was injected into the same GC–MS system mentioned above, which was equipped with a chiral Cyclosil-B capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies Ltd., CA, USA). The temperature program was as follows: hold at 60 °C for 1 min, increase from 60 to 160 °C at a rate of 4 °C/min, followed by increase from 160 to 240 °C at a rate of 20 °C/min with final isothermal hold at 240 °C for 5 min. Helium was used as the carrier gas at a flow rate of 2 mL/min with a split ratio of 1:10, the injector temperature was 200 °C, and the detector temperature was 220 °C. Mass spectra were recorded over a 40–400 amu range at 3.3 scans/s with an ionization energy of 70 eV. The percentage of enantiomeric excess (% ee) of (*R*)-(−)- and (*S*)-(+)-linalool was calculated using the peak area of the enantiomer peaks in the GC–MS total ion current chromatogram.

Isolation and identification of phenolic compounds

About 100 and 70 g of fresh *L. umbellata* leaves and twigs (pooled, not individual), respectively, were extracted using a 70% (v/v) aqueous acetone solution three times each at room temperature (ca. 25 °C) for 15 h. The extract solutions were evaporated and freeze dried, to give 13.2 g and 5.5 g of leaf and twig extracts, respectively. These extracts were further extracted with ethyl acetate, and the ethyl acetate extracts were then re-extracted with a

70% aqueous methanol solution. The yields of the 70% methanol extracts of leaves and twigs were 1.5 and 1.1 g, respectively. Subsequently, the 70% methanol extracts of leaves (1.2 g) were dissolved in 70% aqueous methanol, to isolate compounds by preparative high-performance liquid chromatography (HPLC) (LC–VP system; Shimadzu Co., Ltd., Kyoto, Japan) using the following conditions: column, L-column2 ODS (5 μm, 250 mm × 20 mm i.d., CERI); column temperature, 40 °C; mobile phase A, 1% (v/v) acetic acid aqueous solution; mobile phase B, acetonitrile; gradient condition, 60–100% (v/v) of B (0–40 min, linear); flow rate, 5 ml/min; and detection, ultraviolet (UV) at 280 and 320 nm. Several compounds were isolated and analyzed on a nuclear magnetic resonance (NMR) spectrometer (AVANCE 400 III HD, Bruker Ltd., Billerica, MA, USA) and a fast atom bombardment–mass spectrometry (FAB–MS) instrument (HX-110A, JEOL Ltd., Tokyo, Japan). Four compounds were identified as pinocembrin chalcone (8.8 mg) [16], pinocembrin (17.7 mg) [17], pinostrobin chalcone (8.8 mg) [18], and pinostrobin (17.7 mg) [19] based on a comparison with the literature values. Similarly, another compound that was isolated from the 70% methanol extracts of twigs (1.1 g) was identified as 5,6-dehydrokawain (9.9 mg) [20]. The FAB–MS and set of NMR data used for the identification of phenolic compounds (Additional file 1: Fig. S1) were attached as Additional file Information.

Quantification of flavonoids

One gram of each milled sample was steeped in 100 mL of a 70% (v/v) acetone solution, and the resulting solution was kept at room temperature for 24 h. First, the total phenolic content of this solution was measured via the Folin–Ciocalteu method [21] using catechin as a standard reference. Second, 5 μL of the filtered supernatant was injected into an HPLC system (Prominence system; Shimadzu Co., Ltd., Kyoto, Japan). A quantitative analysis of the 70% acetone extract solution was performed using the following conditions: column, L-column2 ODS (3 μm, 150 × 4.6 mm i.d., CERI); column temperature, 40 °C; mobile phase A, 10 mM H_3PO_4 aqueous solution; mobile phase B, acetonitrile; gradient condition, 10–100% (v/v) of B (0–30 min, linear); flow rate, 1 mL/min; and detection, UV at 280 and 320 nm.

Four-point calibration curves were constructed prior to the analysis of the samples based on the purity (%) and peak area of the five isolated compounds, which included pinocembrin chalcone (72.0%), pinocembrin (76.5%), pinostrobin chalcone (80.4%), pinostrobin (92.2%), and 5,6-dehydrokawain (42.3%). The analysis was performed using the HPLC–UV conditions described above, with the exception of the UV wavelength, which was 280 nm for chalcones and 5,6-dehydrokawain and 320 nm for the remaining compounds. The purity of each isolated compound was measured via a quantitative NMR process [22] using the NMR spectrometer mentioned above, with dimethyl sulfone as an IS and acetone-d_6 as a solvent. Subsequently, the contents of the five major phenolic compounds were calculated from the peak areas using a calibration curve prepared from each isolated compound. These quantifications were performed in triplicate for each sample. The calculated amounts of the four constituents were converted into the dry weight percentage of the actual sample weight (100%).

Statistical analysis

Pearson's correlation coefficient (r) and the P value (P) between each identified flavonoid content were calculated and compared using SPSS Statistics Base 28.0 (IBM Corp., USA), with significance set at $P < 0.05$ or $P < 0.01$. Interpretations of absolute value of r were defined as follows: very weak correlation (0.0 to < 0.2), weak correlation (0.2 to < 0.4), moderate correlation (0.4 to < 0.6), strong correlation (0.6 to < 0.8), and very strong correlation (0.8 to 1.0). The mean and standard deviation (SD) of the inter- and intra-individual coefficient of variation (CV) for the major terpenoid and flavonoid components were compared. The inter-individual CV was calculated among the 15 samples, and the intra-individual CV was assessed among 3 months (June, August, and October).

Results and discussion

Individual variation in major terpenoids

Figure 3 shows an annotated example of the GC–MS analysis of the *n*-hexane extracts from the leaves and twigs of *L. umbellata*. A qualitative analysis showed that peaks for 1,8-cineole and linalool were strongly detected in leaves, followed by terpinen-4-ol and α-terpineol. Conversely, peaks for linalool and geraniol were strongly detected in twigs, and geranyl acetate was characteristically detected in some individuals. The abundance of these oxygenated monoterpenes agreed with the results of a previous *L. umbellata* essential oil analysis [10]. The composition of these components was similar to that of *L. umbellata* var. *membranacea* [14]; however, some differences were detected, such as the high content of limonene in the leaves and the low content of geraniol in the twigs compared with the results of this study. Reportedly, some essential oils extracted from the leaves of *L. umbellata* contain high amounts of limonene, carvone, and caryophyllene [23, 24]; in contrast, none of the samples used in this study exhibited these characteristics.

The four major terpenoids detected in the leaves and twigs of *L. umbellata*, i.e., linalool, 1,8-cineole, geraniol, and geranyl acetate, were quantitatively integrated in the individuals in June, August, and October (Fig. 4). The

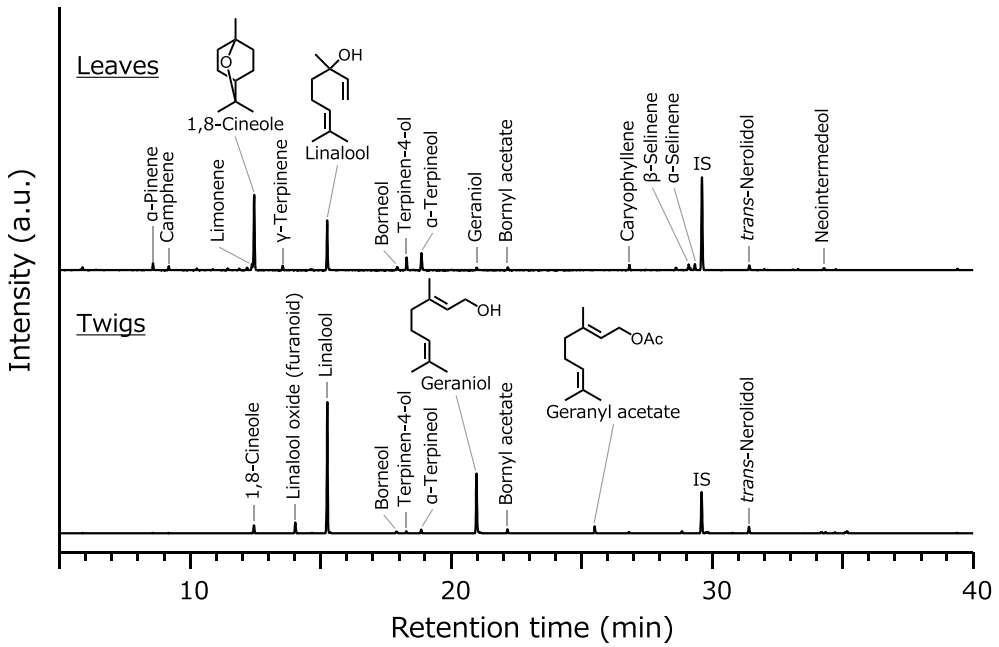

Fig. 3 Annotated example (B1, June) of a GC–MS total ion current chromatogram for *n*-hexane extracts. IS, internal standard (pentadecane)

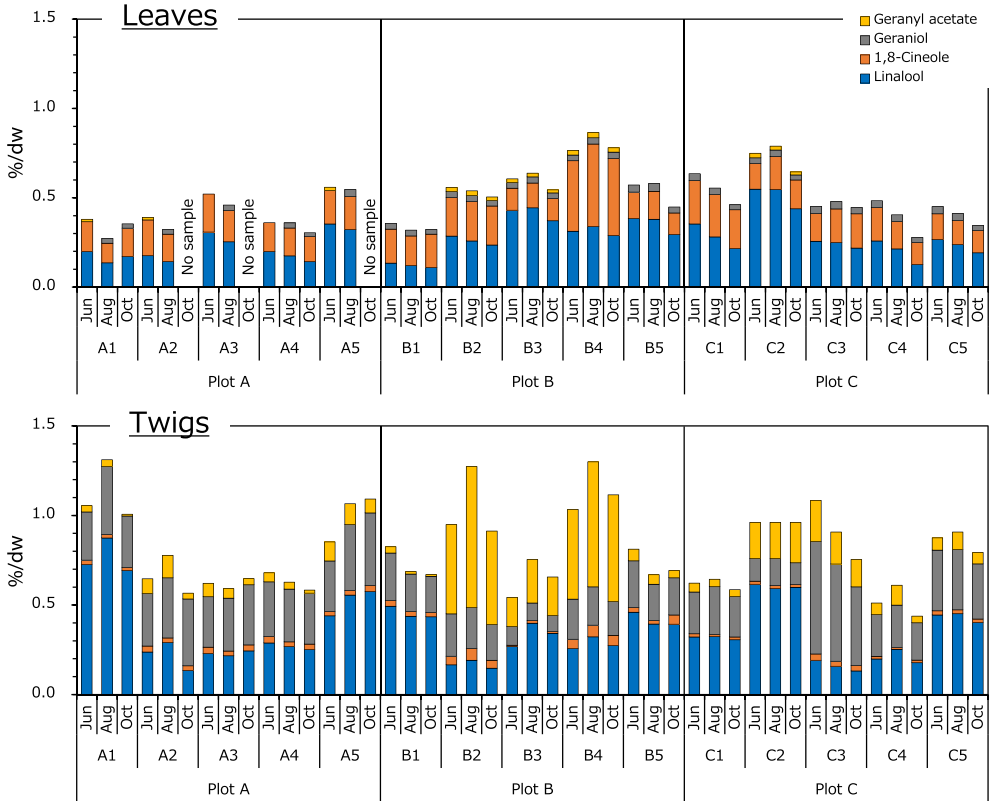

Fig. 4 Quantitative analyses of the four major terpenoids detected in the *n*-hexane extract

results of this analysis showed that the average values of the total components were 0.46%/dw and 0.81%/dw for leaves and twigs, respectively, with the values tending to be higher in the twigs. These findings were in contrast with those of previous reports, in which the leaves contained greater amounts of essential oils than did the twigs [2], which could be attributed to experimental differences, such as sample-drying conditions, regions of sample collection, etc.

The comparison of the content of each component between plots showed no obvious tendency for linalool, 1,8-cineole, and geraniol in both leaves and twigs, indicating that this parameter may be more dependent on individual characteristics than on environmental factors at the growing site. In contrast, geranyl acetate was abundant in the twigs of two individuals in plot B (B2 and B4) at 0.50%/dw–0.79%/dw, and its content was more than twice the sum of the remaining three components regardless of the sample collection season. For the remainder of the individuals in the same plot, there was no synchrony within the plot regarding geranyl acetate content, with detected values of 0.16%/dw–0.24%/dw in B3 and < 0.06%/dw in B1 and B5. These results led us to conclude that the high geranyl acetate detected in some individuals is currently attributed to individual characteristics, rather than an effect of the growth environment.

In all samples, the content of linalool and 1,8-cineole in leaves ranged from 0.11%/dw–0.55%/dw and 0.11%/dw–0.46%/dw, respectively. Meanwhile, the content of linalool and geraniol in twigs ranged from 0.13%/dw to 0.87%/dw and 0.10%/dw to 0.63%/dw, respectively, with slightly larger ranges than those detected in leaves. Pearson's correlation coefficients for the content of the four components in leaves and twigs revealed a strong positive correlation between geranyl acetate and 1,8-cineole in twigs ($r = 0.70$, $P < 0.01$), whereas there were no notable correlations among other components. In other words, the composition of the major terpenoids in *L. umbellata* is likely to be mostly independent in leaves and twigs.

Enantiomeric distribution of linalool

It is well-known that linalool has (*R*)-(−) and (*S*)-(+) enantiomers. Their isomers are widely present in the plant kingdom, and the distribution of enantiomers in essential oils and solvent extracts from various plants has been summarized [25]. Recently, a noteworthy discovery regarding the enantiomers of linalool was reported by a study of *L. umbellata* var. *membranacea*, which identified a high proportion of the (*R*)-(−) and (*S*)-(+) forms in the leaves and twigs, respectively [13]. Similar results have also been reported for the leaves and twigs of *Aniba rosaeodora*, a *Lauraceae* species naturally distributed in the Central Amazon region [26]. However, none of those studies provided any information at the individual level.

The enantiomeric excesses of linalool observed in the 15 individuals of *L. umbellata* analyzed here were calculated based on the results of a GC–MS analysis using a chiral column (Fig. 5). These data showed that the enantiomeric excesses of (*R*)-(−)-linalool in the leaves of all individuals were remarkably high (84–95%). Conversely, although (*S*)-(+)-linalool was predominant in the twigs of most individuals, there was a wide range (35–97%) in its enantiomeric excesses, and one individual in each plot (A3, B2, and C3) contained a high proportion of (*R*)-(−)-linalool. The seasonal differences in enantiomeric excess were very small, within 5% in leaves and within 10% in twigs, with the exception of two individuals (A2 and B2) that showed a peculiar rate. This implies that the enantiomeric excesses of linalool in the leaves and twigs of *L. umbellata* fluctuate very little, at least between June and October, which strongly suggests that it may remain constant across different individuals. The enantiomeric

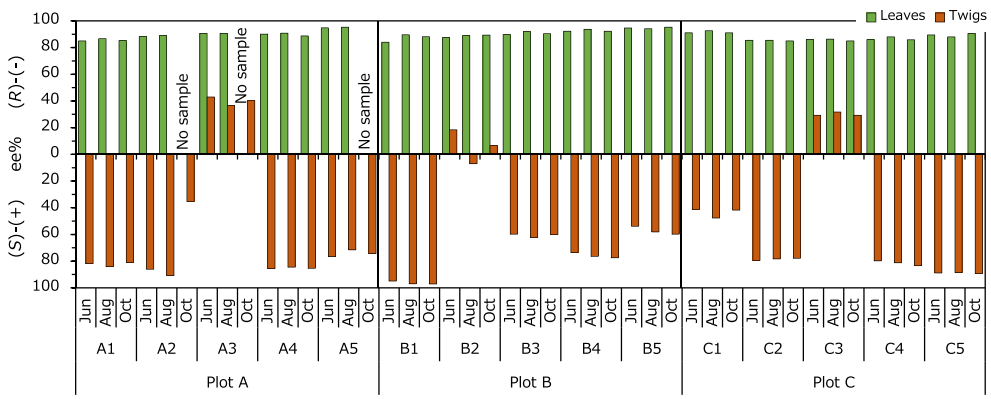

Fig. 5 Enantiomeric excesses (ee) of (*R*)-(−)- and (*S*)-(+)-linalool in the *n*-hexane extract

excesses of other monoterpenoids were as follows: (−)-α-pinene, 38–52% in leaves and 10–49% in twigs; (−)-terpinen-4-ol, 59–65% in leaves and 29–70% in twigs; and (−)-α-terpineol, 76–85% in leaves and 53–81% in twigs (data not shown). Moreover, the range of variation showed that their enantiomeric excesses were more stable in leaves than in twigs, similar to linalool.

Although a slight decrease in the enantiomeric excess of (*R*)-(−)-linalool has been reported under acidic solvents [25], the degree of this decrease does not provide an argument to explain the inter-individual variation in (*S*)-(+)-linalool detected in the twigs. Therefore, it can be presumed that this variation is affected to a greater extent by biotic factors than it is by abiotic factors, i.e., by linalool synthases as enantiospecific enzymes [27]. The enantiomers of linalool, which is abundant in the leaves and twigs of *L. umbellata*, are well-known to differ in scent and bioactivity [28, 29]; therefore, our results provide important information for resource utilization based on the chemical properties of these components.

Individual variation in major flavonoids

Figure 6 shows the total phenolic content of the 70% acetone extracts of *L. umbellata* leaves and twigs. The average total phenolic content was 8.5%/dw in leaves and 3.5%/dw in twigs, indicating that the leaves contained more than twice as much phenols as did the twigs. In leaves, there was variation from ca. 6%/dw (C1) to ca. 11%/dw (B3 and B4), with large differences between individuals. In contrast, twigs showed less variation among individuals and seasons, with stable values of 3%/dw–4%/dw. Although very few studies have compared the total phenolic content of leaves and twigs in related species, a similar result has been reported regarding the total phenolic content in the leaves of *Cinnamomum camphora* (*Lauraceae*), which is about twice as high as that detected in twigs [30].

Figure 7 shows an annotated example of the HPLC analysis of the 70% acetone extracts of the leaves and twigs of *L. umbellata*. The main peaks were fractionated by preparative HPLC and the structures of the isolated compounds were analyzed by NMR. As a result, pinocembrin chalcone, pinocembrin, pinostrobin chalcone, pinostrobin, and 5,6-dehydrokawain were identified as the major low-molecular-weight phenolics in these materials. Four of these compounds, with the exception of pinocembrin chalcone, had been isolated from the bark of *L. umbellata* [31, 32]. Furthermore, there are no reports of the isolation of pinocembrin chalcone from *L. umbellata*. Pinocembrin chalcone has been reported to be a precursor of pinostrobin chalcone and pinocembrin in the general biosynthetic pathway of flavonoids [33]; therefore, it is reasonable to assume that *L. umbellata* commonly contains this compound.

The five phenolic compounds detected in the leaves and twigs of *L. umbellata*, i.e., pinocembrin chalcone, pinocembrin, pinostrobin chalcone, pinostrobin, and 5,6-dehydrokawain, were quantitatively integrated in the individuals in June, August, and October (Fig. 8). The results of this analysis showed that the average value of these total amounts was 0.85%/dw (10%/total phenolic content) and 0.64%/dw (17%/total phenolic content) for leaves and twigs, respectively. Comparisons according to the collection season revealed that the total amounts in both leaves and twigs tended to be lower in October. Moreover, the comparison of content according to chemical structure showed that chalcones were more abundant in leaves and flavanones were more abundant in twigs. Furthermore, 5,6-dehydrokawain was only found in the twigs. In addition to these five compounds, various other phenolic compounds have been reported in *L. umbellata*

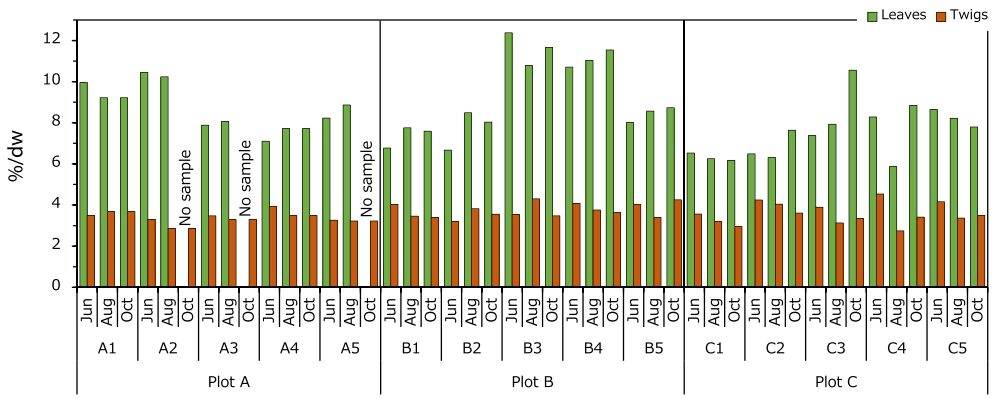

Fig. 6 Total phenol content in the 70% acetone extract

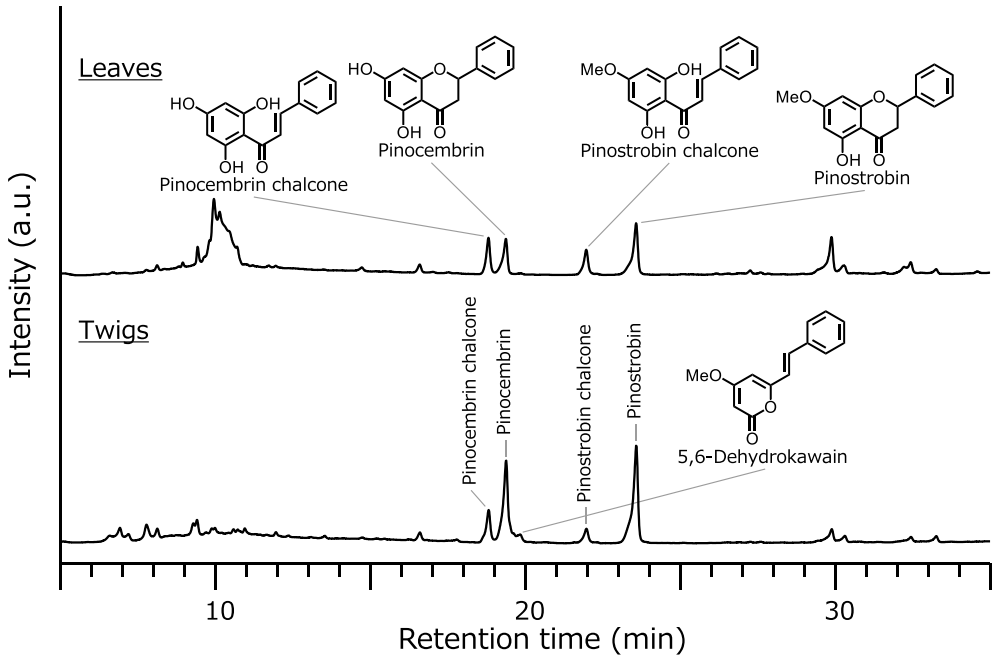

Fig. 7 Annotated example (B1, June) of an HPLC chromatogram from the 70% acetone extract

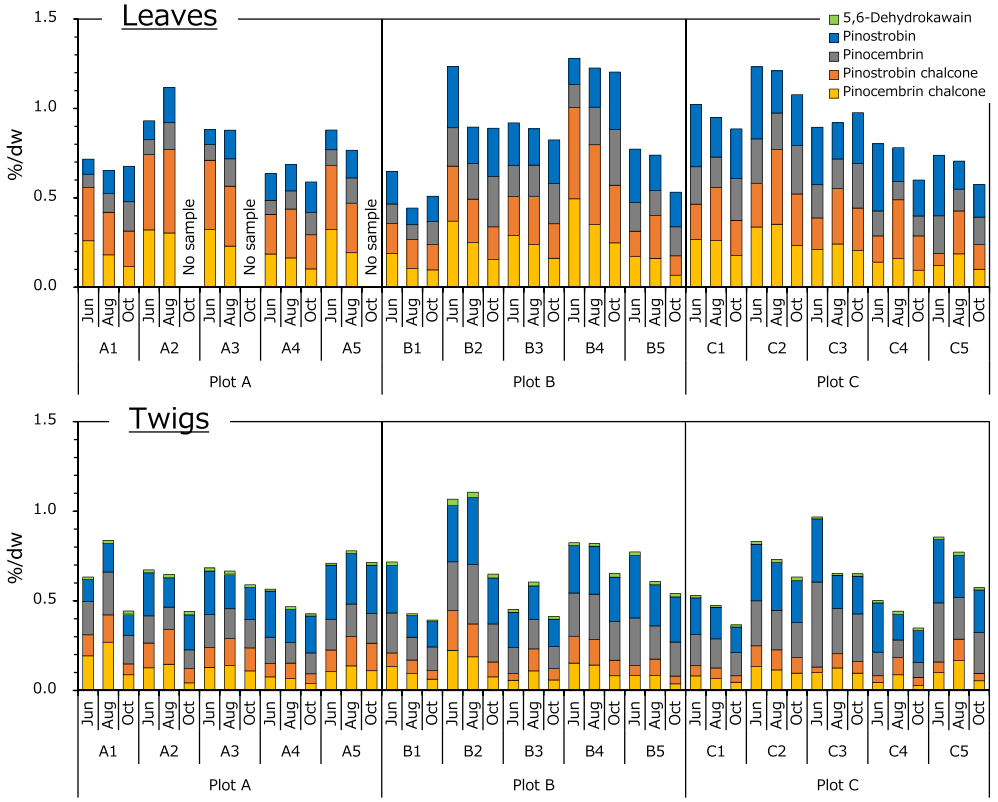

Fig. 8 Quantitative analyses of the five major flavonoids detected in the 70% acetone extract

[31, 34–38]. Therefore, it was expected that the total phenolic content would include these components.

The comparison of the content of each component between plots showed no tendency for the presence of isolated compounds in both leaves and twigs. With the exception of 5,6-dehydrokawain (0.01%/dw–0.03%/dw), the content of pinocembrin chalcone was 0.07%/dw–0.49%/dw in leaves and 0.03%/dw–0.27%/dw in twigs, that of pinostrobin chalcone was 0.07%/dw–0.47%/dw in leaves and 0.03%/dw–0.22%/dw in twigs, that of pinocembrin was 0.08%/dw–0.31%/dw in leaves and 0.01%/dw–0.47%/dw in twigs, and that of pinostrobin was 0.08%/dw–0.40%/dw in leaves and 0.12%/dw–0.37%/dw in twigs. Therefore, the correlations between the content of these four components in leaves and twigs were discussed based on the general biosynthetic pathways of flavonoids and Pearson's correlation coefficients (Fig. 9). These chalcones and flavanones are generally regarded as being derived from the biosynthetic pathway shown in Fig. 9a [33, 39]. The correlation coefficients between each component were calculated for leaves and twigs, respectively, and revealed a very strong or strong positive correlation between pinocembrin chalcone and pinostrobin chalcone in both leaves ($r=0.82$, $P<0.01$) and twigs ($r=0.79$, $P<0.01$) (Fig. 9b). Similarly, a strong positive correlation was found between pinocembrin and pinostrobin in both leaves ($r=0.77$, $P<0.01$) and twigs ($r=0.69$, $P<0.01$) (Fig. 9g). A moderate positive correlation was found between pinocembrin and pinocembrin chalcone in twigs ($r=0.43$, $P<0.01$) (Fig. 9c). A very weak or weak correlations were observed for the remaining combinations (Fig. 9d–f). This implies that strong correlations were observed between the content of pinocembrin chalcone and pinostrobin chalcone, as well as pinocembrin and pinostrobin, which are considered to be in a precursor-derivative relationship via methyl transferase (MT) in the leaves and twigs of *L. umbellata*. Conversely, weak correlations were observed between the content of chalcones and each of the flavanone derivatives via chalcone isomerase (CHI). The relationship between each synthase and content is currently unknown, but these results represent the first report of the flavonoid content in the leaves and twigs of *L. umbellata*.

Inter- and intra-individual differences in major components

Based on the quantification of the four terpenoids and four flavonoids (Figs. 4 and 8) that are abundant in the leaves and twigs of *L. umbellata*, we examined which factor regarding inter- and intra-individual (i.e., interseasonal) differences had a stronger CV (Table 1). For terpenoids, the inter- and intra-individual differences for both leaves and twigs were 40% ± 2% to 49% ± 2% and 10% ± 6% to 14% ± 7%, respectively, for three components (geraniol, linalool, 1,8-cineole), with a higher CV detected for the inter-individual differences. Geranyl acetate in twigs showed a characteristically high CV regarding inter-individual differences (121% ± 16%). Conversely, for flavonoids, the inter-individual differences were slightly higher than the intra-individual differences in all cases, but the differences were relatively small compared with the results obtained for terpenoids. These findings suggest that the content of the major terpenoids in *L. umbellata* leaves and twigs may be more strongly dominated by individual-specific chemical properties than by intra-individual differences caused by variations in collection season. Seasonal differences in the content of essential oils in the leaves and twigs have been studied for linalool [2], and the possibility of variation in both leaves and twigs has been reported when individuals were not distinguished. Nevertheless, the authors also mentioned the possibility of inter-individual differences, and our results strongly supported their speculation. The major flavonoids showed different results from terpenoids, suggesting that these components may be affected by multiple factors, including inter- and intra-individual differences. As most of the *L. umbellata* individuals at the sample collection sites used in this study are deciduous from October to November, it is highly feasible to use these results as a reference for the chemical properties of the leaves. In contrast, there is a lack of data on the chemical properties of twigs from autumn to spring; therefore, further continuous research is needed to clarify the intra-individual variation in the components of twigs.

Conclusions

In this study, inter- and intra-individual differences in the major terpenoids and flavonoids in native *L. umbellata* from the same region were investigated in detail at the individual level. The new findings on terpenoids included the observation that the compositions of the major monoterpenoids were strongly dependent on the properties of each individual, that the composition was generally independent of leaves and twigs, and that geranyl acetate was characteristically present in the twigs of some individuals. Moreover, the new findings regarding linalool included the observation that, although most individuals showed similar enantiomeric properties to those of *L. umbellata* var. *membranacea* [13], there were a few individuals with a high (*R*)-(−)-linalool content in the twigs, and the proportion of enantiomers remained constant in each individual, regardless of the season. The new findings on flavonoids included the observation that the total phenolic content of leaves was more than twice that of twigs,

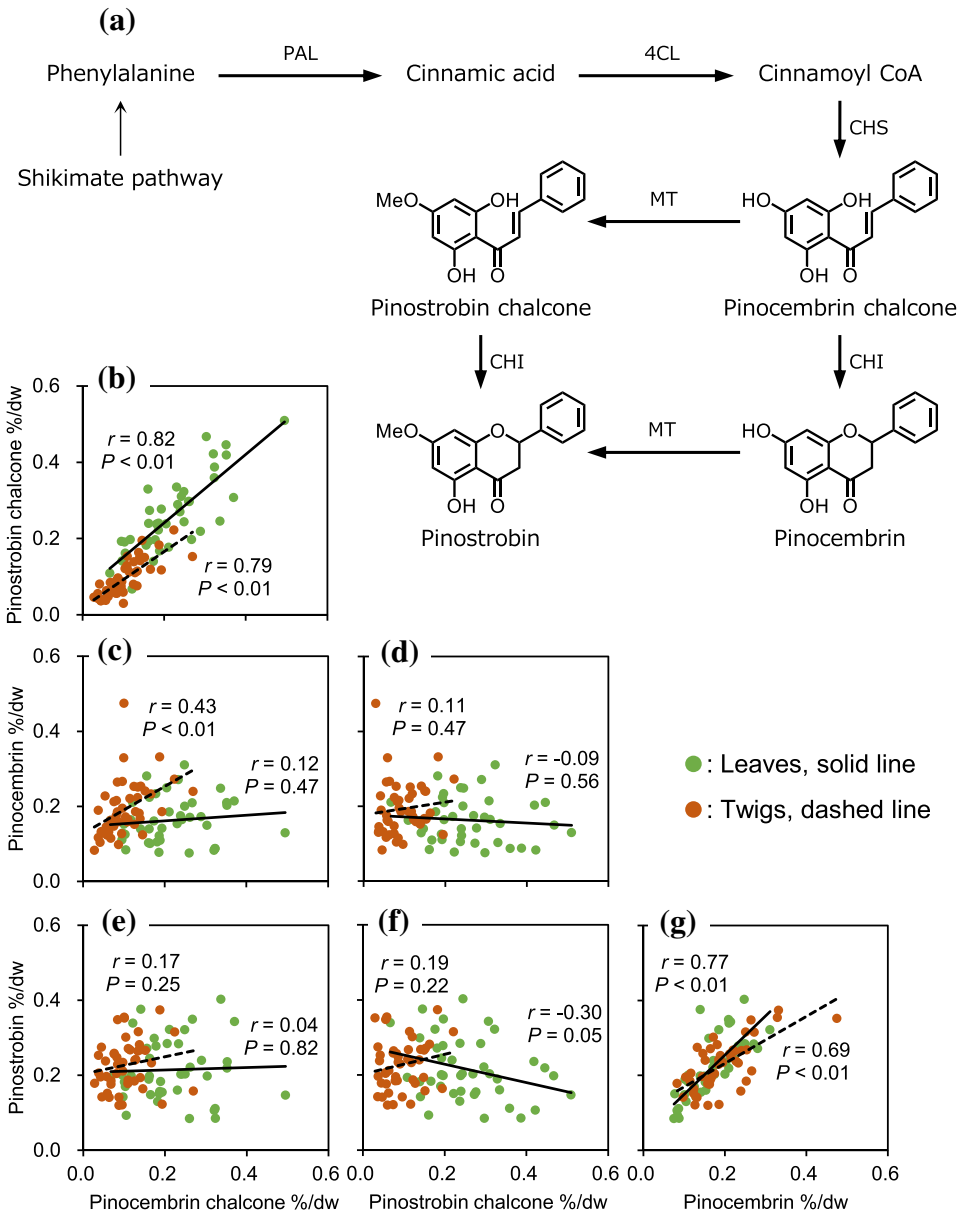

Fig. 9 General biosynthetic pathway for flavonoids and Pearson's correlation coefficient and *P* value between each component. **a** General biosynthetic pathway of the identified flavonoids, **b** pinocembrin chalcone vs. pinostrobin chalcone, **c** pinocembrin chalcone vs. pinocembrin, **d** pinostrobin chalcone vs. pinocembrin, **e** pinocembrin chalcone vs. pinostrobin, **f** pinostrobin chalcone vs. pinostrobin, **g** pinocembrin vs. pinostrobin. *r*, correlation coefficient; *P*, *P* value; *PAL* phenylalanine ammonia lyase, *4CL* 4-coumarate: CoA ligase, *CHS* chalcone synthase, *CHI* chalcone isomerase, *MT* methyl transferase

that the leaves tended to contain more chalcones and twigs more flavanones, and that the content between chalcones (pinocembrin chalcone vs. pinostrobin chalcone) and between flavanones (pinocembrin vs. pinostrobin) was positively correlated in both leaves and twigs. Furthermore, the CV calculated from the results of the quantitative analysis revealed that the content of the major terpenoids was determined by inter-individual, rather than intra-individual, differences, and that the content of the major flavonoids could be affected by multiple factors, including inter- and intra-individual differences. Although several intriguing results were obtained, this study should at present only be applicable to a limited population native to specific regions.

Table 1 Mean values of the inter- and intra-individual coefficient of variation for the major terpenoids and flavonoids

Components	Leaves (%)		Twigs (%)	
	Inter[a]	Intra[b]	Inter[a]	Intra[b]
Terpenoids				
Geranyl acetate	–	–	121 ± 16	33 ± 19
Geraniol	–	–	40 ± 2	11 ± 5
Linalool	41 ± 4	14 ± 7	49 ± 2	12 ± 9
1,8-Cineole	42 ± 7	10 ± 6	–	–
Flavonoids				
Pinocembrin chalcone	37 ± 5	28 ± 11	41 ± 1	35 ± 15
Pinocembrin	34 ± 7	25 ± 11	35 ± 5	19 ± 9
Pinostrobin chalcone	35 ± 10	25 ± 14	46 ± 11	35 ± 16
Pinostrobin	32 ± 15	30 ± 12	26 ± 4	21 ± 11

[a] CV (± SD) of the inter-individual variations calculated using the CV of each measured content (%/dw) over three seasons (June, August, and October)

[b] CV (± SD) of the intra-individual variations calculated using the CV of each measured component (%/dw) in 15 individuals (A1–5, B1–5, and C1–C5). Components with a maximum content of < 0.1%/dw in each plant part are hyphenated

Nevertheless, our findings provide key knowledge in considering the sustainable use of *L. umbellata*, including the advancement of cultivation techniques and their effective use based on the chemical characteristics of the resource.

Abbreviations

GC–MS: Gas chromatography–mass spectrometry; HPLC: High-performance liquid chromatography; UV: Ultraviolet; NMR: Nuclear magnetic resonance; FAB–MS: Fast atom bombardment–mass spectrometry; SD: Standard deviation; CV: Coefficient of variation; MT: Methyl transferase; CHI: Chalcone isomerase.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s10086-022-02066-x.

Additional file 1. Figure. S1 Chemical structures of identified phenolic compounds.

Acknowledgements

The authors thank Mr. Kouzou Hirata (Kuromoji craftsman) and Mr. Takao Mori (Ujyou-youji, Traditional crafts officially designated by Chiba Prefecture) for the grateful supports on the sample collections and identifications.

Author contributions

All of the authors listed in this work provided academic contributions to the development of this manuscript. NK designed and integrated this study, collected samples, performed GC–MS analyses, interpreted the results and drafted the manuscript. TM participated in the GC–MS analyses, interpreted the statistically analyzed data and drafted the manuscript. KH participated in the design of this study, collected samples, performed HPLC and NMR analyses and drafted the manuscript. NM participated in the design of the study, collected samples and interpreted the GC–MS data. TO participated in the design of this study, interpreted the data and supervised the final manuscript. All authors have read and agreed to the published version of the manuscript.

Funding

This study was funded by Research Grant #201806 of the Forestry and Forest Products Research Institute.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests

The authors declare that they have no competing interests.

Received: 8 July 2022 Accepted: 6 November 2022
Published online: 21 November 2022

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