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Accessing the natural variation of the abundances of major lignans in the heartwood of *Taiwania cryptomerioides* by ^{1}H-NMR and LC-MS profiling

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Abstract: Lignans are major bioactive secondary metabolites, which are also formed in the heartwood (hW) of Taiwania (*Taiwania cryptomerioides*). Their biosynthesis pathways are complex and involve many enzymes and intermediates. To evaluate the extent of the genetic components leading to the variety of lignans in Taiwania hW, 35 Taiwania genotypes of four provenances were surveyed using the proton nuclear magnetic resonance (^{1}H-NMR) and liquid chromatography-mass spectrometry (LC-MS) analyses. The metabolite profiles were statistically evaluated by principal component analysis (PCA) and the general linear model (GLM). The broad-sense heritability (H^2) was further evaluated by linear mixed model (LMM) analysis. It was demonstrated that the genetic factor is the major contributor to the abundance of lignans, though the environmental factor also has some effect on it. Among the metabolites detected by ^{1}H-NMR, lignans were the major compounds that exhibited high a H^2 (0.52–0.82), which was further verified by LC-MS. The conclusion is that ^{1}H-NMR spectroscopy is suitable for quick screenings, predictions and semi-quantitation of lignans. The high H^2 is also indicative of the lignan abundances as traits that can be genetically modified to achieve a significant wood quality improvement.

Keywords: ^{1}H-NMR, broad-sense heritability, heartwood, LC-MS, lignan, metabolomics analysis, natural variation, *Taiwania cryptomerioides*

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Introduction

Taiwania (*Taiwania cryptomerioides* Hayata) is a conifer species endemic to Taiwan. It is a relatively fast-growing tree and can often be recognized by the height that outgrows the neighboring trees. Taiwania is sparsely scattered at high elevations (from 1800 to 2600 m above sea level) in Taiwan, and at the mountains around the border between Myanmar and China. Taiwania is unique because of its monotypic genus status. Because of its exceptional wood qualities, it is one of the most valuable gymnosperm species in Taiwan.

Hundreds of terpenoids, lignans, flavonoids and steroids have been identified from the root, bark, wood and needles of Taiwania (Chang et al. 2003; Chien and Kuo 2009). Many identified extractives contribute to the photodiscoloration and durability in Taiwania heartwood (hW) (Chang et al. 1998, 1999a,b, 2001), among which lignans are the most abundant (Tsao et al. 2016). Lignans arise via complex biosynthesis pathways with the participation of many enzymes and intermediates (Suzuki and Umezawa 2007; Tsao et al. 2016; Chiang et al. 2018) as illustrated in Figure 1.

Lignans display antiviral, anticancer, anti-inflammatory, anti-oxidative, immunosuppressive and hepatoprotective activities (Adlercreutz 2007; Shyur et al. 2010), and also play an important role in plant defense and in plant growth regulation. For example, hinokinin helps avoid chromosome damages (Medola et al. 2007), savinin has anti-inflammatory and antiviral activities (Cho et al. 2001; Wen et al. 2007), taiwanin A shows anticancer activity (Ho et al. 2007, 2012a,b; Shyur et al. 2010; Harn et al. 2014), taiwanin C inhibits prostaglandin E2 (PGE2) formation (Ban et al. 2002), taiwanin E inhibits cancer cell migration and anti-proliferation (Wang et al. 2014; Hsu et al. 2017), and helioxanthin has anticancer and antiviral activities (Tseng et al. 2008a,b; Lin et al. 2016). The essential oils of Taiwania smell good and can inhibit the growth of fungi, bacteria and tumor cells (Chang et al. 1999b, 2000a,b, 2001, 2003; Ho et al. 2012b).

Metabolomic analysis is a rapidly developing research field which had been widely applied to understand the

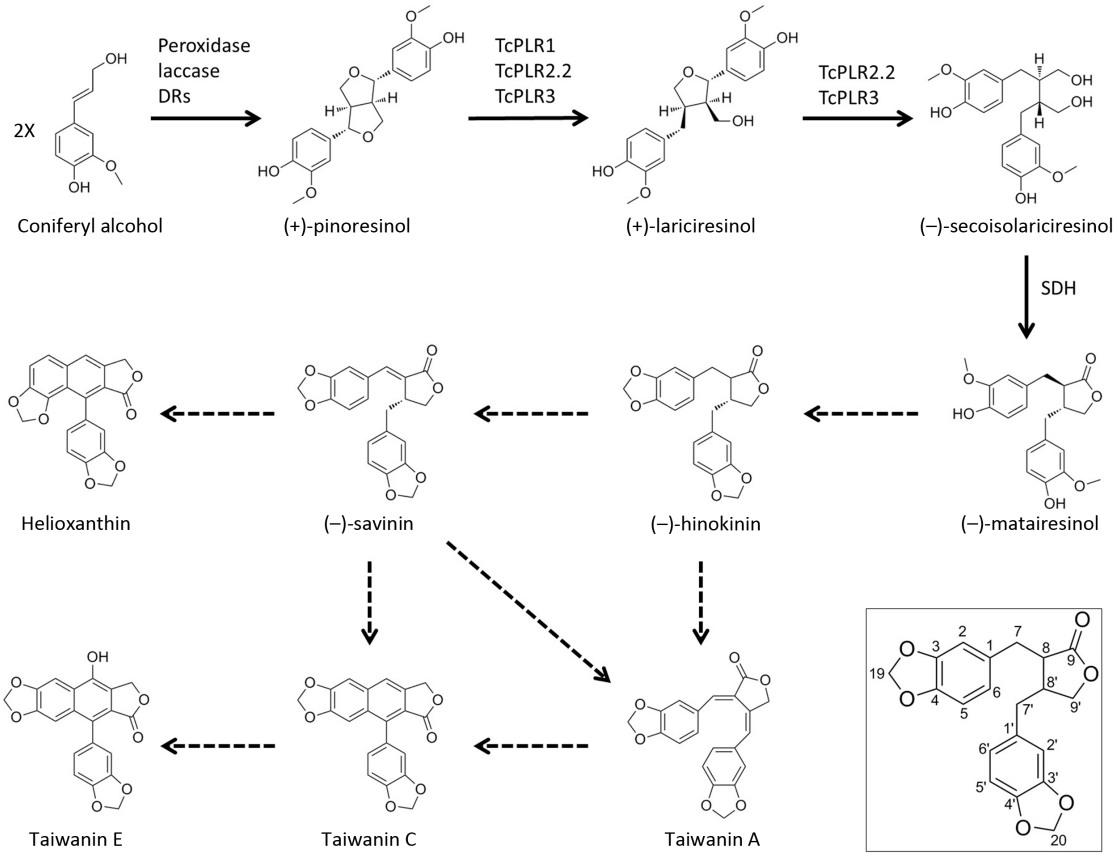

Figure 1: A hypothesized lignan biosynthesis pathway in the heartwood of Taiwania adapted from Tsao et al. (2016). The structure in the box indicates the carbon position of the lignans.

composition and biosynthesis of plant secondly metabolites (Lambert et al. 2007a,b; Shi et al. 2013; Chandradevan and Bala 2014; Clausen et al. 2014; Fraige et al. 2014; Pan et al. 2015; Zhao et al. 2016). Specific metabolites revealed by the analysis can be studied further to understand the genetic mechanisms behind the biosynthesis (Hsieh et al. 2015; Hsu and Chu 2015; Chiang et al. 2018).

Heritability is a measurement of the genetic variation in a population and it provides an estimate of how the traits respond to the selection in a population. In forest trees, the growth, height, diameter, physical and chemical properties of wood such as density, microfibril angle (MFA), lignin and cellulose content, are considered as valuable traits targeted for genetic improvement (Hannrup et al. 2004; Poke et al. 2006; Ukraninetz et al. 2008; Porth et al. 2013; Hall et al. 2016). The metabolite content is considered as a quantitative trait and is analyzed in the context of human (Alul et al. 2013; Demirkan et al. 2015) and agricultural studies (Li et al. 2016; Matros et al. 2017). Monosaccharides in the Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) (Robinson et al. 2007) and resins in *Pinus elliottii* Engelm. (Lai et al. 2017), and other metabolites in *Pinus taeda* L. (Eckert et al. 2012) were studied as quantitative traits aimed at the genetic improvement of growth and metabolite contents. There is a wide range of narrow-sense heritability for different metabolites, suggesting that the variations of some metabolites may be affected by the biosynthesis pathways or the tissue sources that are susceptible to environmental effects.

In the present study, the natural variation of the hW metabolite contents were evaluated, with the focus on 35 Taiwania genotypes of four provenances and on their six major constituents (hinokinin, savinin, taiwanin A, taiwanin C, taiwanin E and helioxanthin, see Figure 1). The proton nuclear magnetic resonance (^{1}H-NMR) and liquid chromatography-mass spectrometry (LC-MS) approaches were selected for analysis. ^{1}H-NMR spectroscopy of the complex extractive mixture (without separation of its individual components) is a rapid method and is well suited for screening experiments, and which reveals simultaneously the presence of primary and secondary metabolites and their concentrations. However, less abundant compounds cannot be detected by this approach, and the evaluation of the spectra becomes difficult in the case of very complex mixtures. LC-MS is more specific and has

a higher sensitivity even for minor compounds. Analysis with different columns increases the analytical power of LC-MS. The statistical methods such as principal component analysis (PCA), the general linear model (GLM) and the linear mixed model (LMM) were applied for data evaluation and the broad-sense heritability (H^2) of each metabolite profile were presented, while the genetic and environmental effects were differentiated.

Materials and methods

Plant materials: Taiwania (*T. cryptomerioides*) heartwood (hW) samples were collected from the Tsuyunshan seed orchard, which was established in 1981 with a collection of 35 genotypes of four provenances. There were 17 genotypes (1–17) from Guanshan provenance; four genotypes (18–21) from Jhudong provenance; six genotypes (22–27) from Tasheueshan provenance; and eight genotypes (28–35) from Dajia provenance. In the original seed orchard design, each of the genotypes was replicated with 14–20 ramets and arranged in a complete random block design. However, the original plot design was mostly disrupted due to disease or weather damage over the past 35 years. The remaining trees were re-examined and re-designed in five plots for material sampling (Figure 2). In the new plot design, each genotype was included in at least four plots that represent clonal replicates, and the replicated clonal trees were at least 30 m apart from each other. Wood cores 10 cm long and 0.5 cm in diameter were collected with an increment borer from Taiwania trunk at 130 cm above the ground of the upper hillside. The wood core included sapwood (sW), a transition zone (TZ) and a hW portion of the harvested sample trees. A total of 171 wood samples were collected and the samples of 2 cm section in the outer hW region (Tsao et al. 2016) were grounded under liquid N_2. The milled samples were freeze-dried and extracted with MeOH.

NMR spectra: A Bruker Avance III-400 NMR (Bruker, Billerica, MA, USA) was applied. One milligram of extract was dissolved in 600 µl MeOH-d4 (CD_3OD) containing 0.03% tetramethylsilane (TMS) (Sigma-Aldrich, St. Louis, MO, USA) and placed in 5 mm NMR tubes (Sigma-Aldrich, St. Louis, MO, USA). Spectra were obtained at 300 K without spinning. The ^{1}H-NMR spectra were manually phase-corrected and calibrated to TMS as 0 parts per million (ppm). The spectrum was divided into integrated regions of equal width (0.04 ppm) corresponding to the chemical shift (δ) 8.00–4.96 and δ 2.00–0.48.

LC-MS quantification of the lignans: A Bruker amaZon speed-ion trap mass spectrometer (Bruker, Billerica, MA, USA) coupled with a Thermo Scientific Dionex UltiMate 3000 system (Thermo Fisher Scientific, Waltham, MA, USA) was used (Bruker Hystar software). The separation was performed on a Waters RP-18, 100×2.1 mm, 1.7 micro column. The eluting solvent system was 0.1% (v/v) acetic acid (Sigma-Aldrich, St. Louis, MO, USA) in water (A), MeOH (Sigma-Aldrich, St. Louis, MO, USA) (B) and acetonitrile (Sigma-Aldrich, St. Louis, MO, USA) (C). The gradient started with 40% A and 60% C for 7 min, →30% A, 60% B and 10% C at 9 min, →20% A, 70% B and 10% C at 11 min, →20% A, 65% B and 15% C at 20 min, →100% B at 23 min and held for 5 min. The column temperature was 30°C. The flow rate was 0.1 ml min^{-1} and 3 µl was injected from a 1 mg ml^{-1} concentrated extract in MeOH. The mass parameters setting was as follows: 4.5 kV of capillary; 1.93 bar of nebulizer pressure; 8.0 l min^{-1} of dry gas flow at 250°C. The six lignans (hinokinin, savinin, taiwanin A, taiwanin C, taiwanin E and helioxanthin, Figure 1) were quantified according to Tsao et al. (2016).

Broad-sense heritability (H^2) of metabolite abundance by LMM: The metabolite abundance obtained by NMR and LC-MS were analyzed by the LMM and in terms of H^2 based on the assumption that: $Y_{ij}=u+G_i+E_{ij}$, where: Y_{ij} is the phenotypic value (metabolite abundance) of the j_{th} ramet of the i_{th} ortet. i=1–35, represent the 35 genotypes in the Tsuyunshan seed orchard. j=1–5, represent the five assigned plots of sampling. u is the population mean. G_i is the genetic effect of the i_{th} ortet. E_{ij} is the random environmental effect on the j_{th} ramet of the i_{th} ortet.

The H^2 of the metabolites were calculated by the LMM approach focusing on the corresponding genetic (V_G), environmental (V_E) and phenotypic variances (V_P). The H^2 was calculated according to Wricke and Weber (1986). $H^2=V_G/V_P=V_G/(V_G+V_E/r)$, where r is the five assigned plots of sampling. The correlation analysis was performed by PROC CORR of the Statistical Analysis System (SAS, Cary, NC, USA) statistics software package, with a focus on the significance level of the genotype, provenance and plot effect.

Principal component analysis (PCA): The NMR and LC-MS data were evaluated by the multivariate statistical package (MVSP, Kovach Computing Services, Pentraeth, UK) V3.13m. PCA results were based on Kaiser's and Joliffe's rules (Wang et al. 2006).

Results and discussion

Metabolic profiling by NMR

The ^{1}H-NMR spectra of the 171 outer hW samples were recorded (Figure 3). The ppm range 2.0–0.5 contained the protons of the methyl groups (-CH_3), which are suitable for differentiating different terpenoid skeletons in Taiwania (Chang et al. 2000b; Wang et al. 2002). The signals of 4.5–3.0 ppm were the proton signals in the sugar or the methoxy group (-OCH_3). The proton signals of the aromatic ring in lignans were mostly in the range of 8.0–6.0 ppm. The signals in the ranges of 8.00–4.96 and 2.00–0.48 ppm were integrated in 0.04 ppm intervals, which gave a total of 115 integrated signals (Table 1). These integral signals were analyzed subsequently by PCA. The PCA score plots did not show any cluster according to provenance origins or genotypic differences (data not shown). However, when PCA score plots were grouped based on the five experimental plots (Figure 4), a somewhat different pattern appeared. The samples of plot 1 were clustered more at the bottom, those of plot 2 at the central left corner, and those from plot 3 in two major areas (with samples from Guanshan provenance on the right, and the remaining samples on the left). Experimental plots 4 and 5 were clustered at the central bottom corner and central top corner, respectively. From the

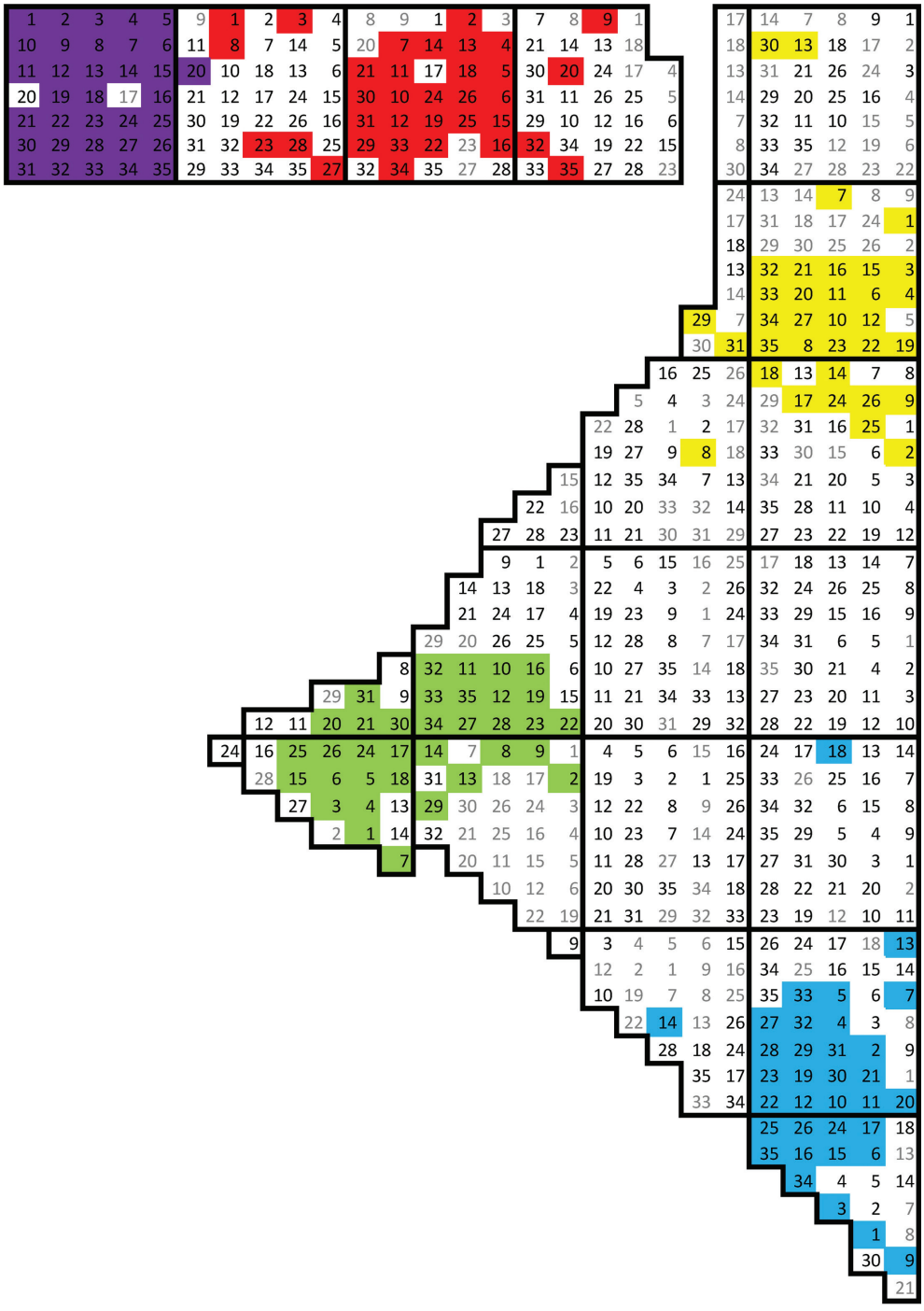

Figure 2: The experimental plot design of 35 Taiwania genotypes in Tsuyunshan seed orchard.
Five experimental plots (purple: plot 1; red: plot 2; yellow: plot 3; green: plot 4; blue: plot 5) were designed as each genotype was represented in at least four experimental plots.

PCA results, it could be interpreted that the secondary metabolites may have been influenced by environmental factors to some extent. According to the loadings, the PCAs between 77 and 109 ppm represent the hydrogens located in the saturated region (Table 1), while terpenoids, steroids and fatty acids showed proton signals in this region.

Lignans LC-MS profiling

The lignans of Taiwania are derived from coniferyl alcohol (CA) via the pathway illustrated in Figure 1. The CA is converted to hinokinin and then further to savinin, helioxanthin and taiwanin A, C and E (Tsao et al. 2016). Recently,

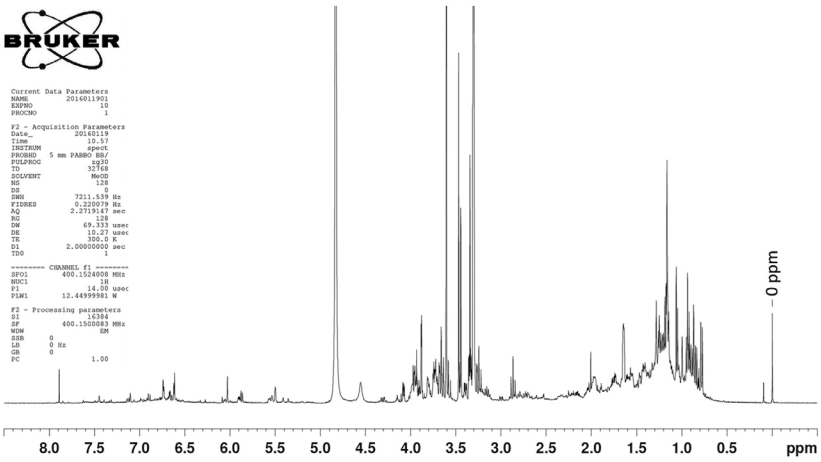

Figure 3: A representative chromatogram of ^{1}H-NMR profiling of the secondary metabolites in the outer heartwood of Taiwania.

Table 1: Integrated signals and their corresponding ^{1}H-NMR chemical shifts.

Integral	Chem. shift (ppm)	Integral	Chem. shift (ppm)	Integral	Chem. shift (ppm)
1	8.00–7.96	40	6.44–6.40	79	1.92–1.88
2	7.96–7.92	41	6.40–6.36	80	1.88–1.84
3	7.92–7.88	42	6.36–6.32	81	1.84–1.80
4	7.88–7.84	43	6.32–6.28	82	1.80–1.76
5	7.84–7.80	44	6.28–6.24	83	1.76–1.72
6	7.80–7.76	45	6.24–6.20	84	1.72–1.68
7	7.76–7.72	46	6.20–6.16	85	1.68–1.64
8	7.72–7.68	47	6.16–6.12	86	1.64–1.60
9	7.68–7.64	48	6.12–6.08	87	1.60–1.56
10	7.64–7.60	49	6.08–6.04	88	1.56–1.52
11	7.60–7.56	50	6.04–6.00	89	1.52–1.48
12	7.56–7.52	51	6.00–5.96	90	1.48–1.44
13	7.52–7.48	52	5.96–5.92	91	1.44–1.40
14	7.48–7.44	53	5.92–5.88	92	1.40–1.36
15	7.44–7.40	54	5.88–5.84	93	1.36–1.32
16	7.40–7.36	55	5.84–5.80	94	1.32–1.28
17	7.36–7.32	56	5.80–5.76	95	1.28–1.24
18	7.32–7.28	57	5.76–5.72	96	1.24–1.20
19	7.28–7.24	58	5.72–5.68	97	1.20–1.16
20	7.24–7.20	59	5.68–5.64	98	1.16–1.12
21	7.20–7.16	60	5.64–5.60	99	1.12–1.08
22	7.16–7.12	61	5.60–5.56	100	1.08–1.04
23	7.12–7.08	62	5.56–5.52	101	1.04–1.00
24	7.08–7.04	63	5.52–5.48	102	1.00–0.96
25	7.04–7.00	64	5.48–5.44	103	0.96–0.92
26	7.00–6.96	65	5.44–5.40	104	0.92–0.88
27	6.96–6.92	66	5.40–5.36	105	0.88–0.84
28	6.92–6.88	67	5.36–5.32	106	0.84–0.80
29	6.88–6.84	68	5.32–5.28	107	0.80–0.76
30	6.84–6.80	69	5.28–5.24	108	0.76–0.72
31	6.80–6.76	70	5.24–5.20	109	0.72–0.68
32	6.76–6.72	71	5.20–5.16	110	0.68–0.64
33	6.72–6.68	72	5.16–5.12	111	0.64–0.60
34	6.68–6.64	73	5.12–5.08	112	0.60–0.56
35	6.64–6.60	74	5.08–5.04	113	0.56–0.52
36	6.60–6.56	75	5.04–5.00	114	0.52–0.48
37	6.56–6.52	76	5.00–4.96	115	0.02 to −0.02
38	6.52–6.48	77	2.00–1.96		
39	6.48–6.44	78	1.96–1.92		

Integral 115 was the internal standard signal (TMS).

^{1}H-NMR, Proton nuclear magnetic resonance; TMS, tetramethylsilane.

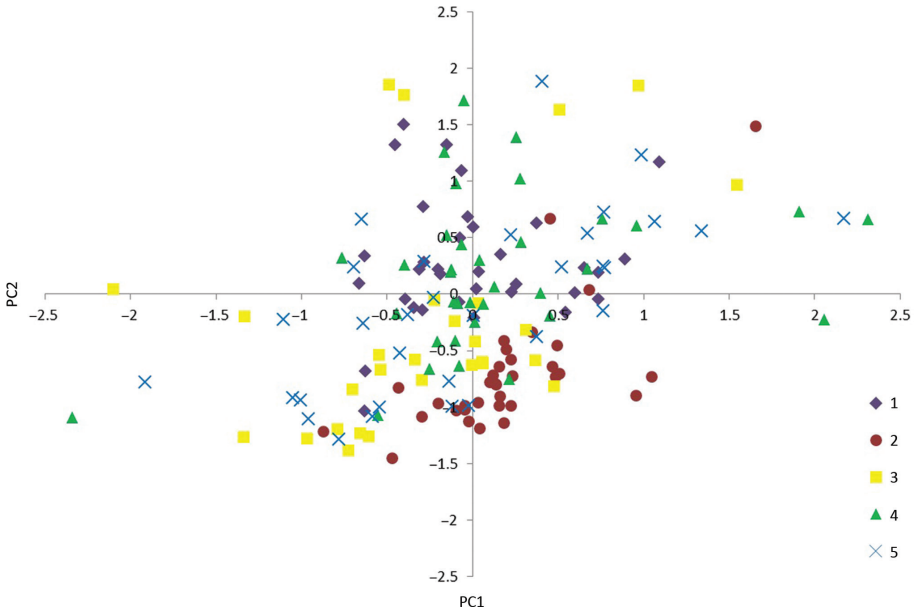

Figure 4: PCA score plot based on the integrated signal of ^{1}H-NMR metabolite profiling of Taiwania outer hW and grouped by experimental plots.

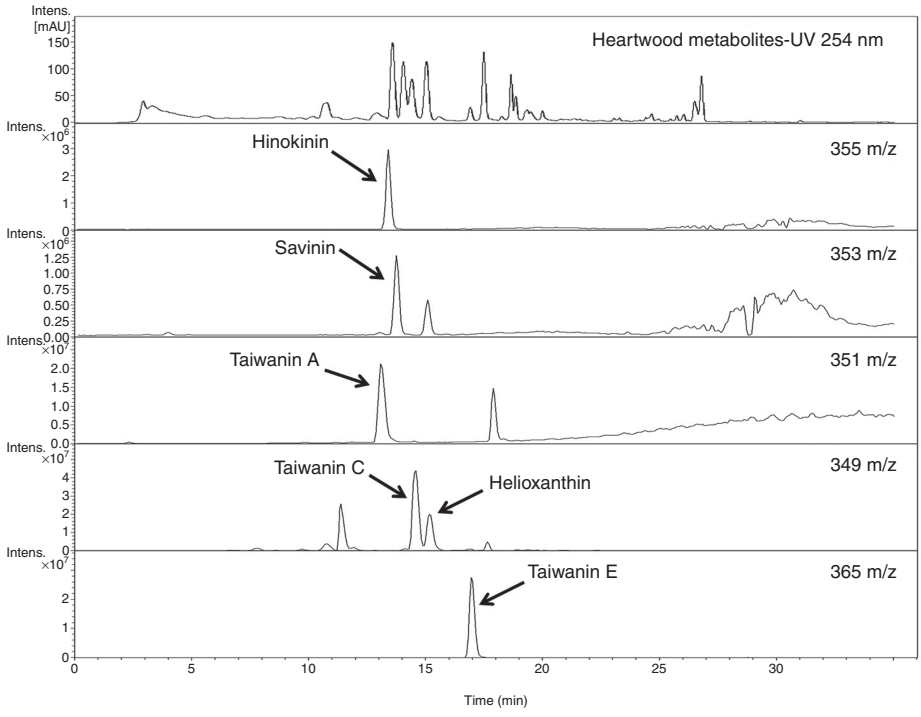

Figure 5: A representative chromatogram of ultra-high-performance liquid chromatography (UHPLC) profiling of the secondary metabolites in the outer hW of Taiwania.

three Taiwania pinoresinol-lariciresinol reductases (PLRs; TcPLR1, TcPLR2.2 and TcPLR3) were identified that convert (+)-pinoresinol to lariciresinol and subsequently to secoisolariciresinol in the upstream pathway (Chiang et al. 2018). The main lignans were separated by LC-MS (Figure 5). The masses 349, 351, 353, 355 or 365 *m/z* were selected to quantify the contents of specific lignans according to Tsao et al. (2016). The PCA score plots of the lignans abundances in the outer hWs were grouped according to the provenance origin (Figure 6a) or to experimental plots (Figure 6b). The PCA score plots did not show a clear clustering pattern according to the provenances (Figure 6a). However, when

the plots were grouped by different experimental plots (Figure 6b), the experimental plot 3 appeared to cluster at the top. The loading plot results indicated that the principal components 1 were taiwanin C and taiwanin E (Table 2). The PCA results suggest that the biosynthesis of lignans might have been affected by the environment to some extent.

Estimation of the H^2 of lignans by LMM

The H^2 was calculated to characterize the source of variation for each integrated NMR signal and lignan content. The genetic component of the variance V_G is based on the 35 genotypes of all the plots, whereas the environmental component of the variance V_E is based on five plots of the clonal trees. When calculating H^2, the V_E were divided by 5 to obtain the average V_E of an individual genotype. The H^2 data of all the 115 integrated ^{1}H-NMR signals (Figure 7) indicate a significantly high H^2 for integrals less than 75. These regions match the proton signals contributed by lignans. Among the 115 integrals, 38 integrals can be contributed by six lignans. The following integrals can be assigned as follows: 14, 22, 23, 28, 33, 34, 50, 53 and 54 to savinin; 33–38 and 53 to hinokinin; 12, 32, 34, 36, 37, 39, 43, 47 and 54–56 to taiwanin A; 4, 26, 27, 31, 32, 48, 49 and 65 to taiwanin C; 10, 27, 28, 32, 33, 49, 50 and 67

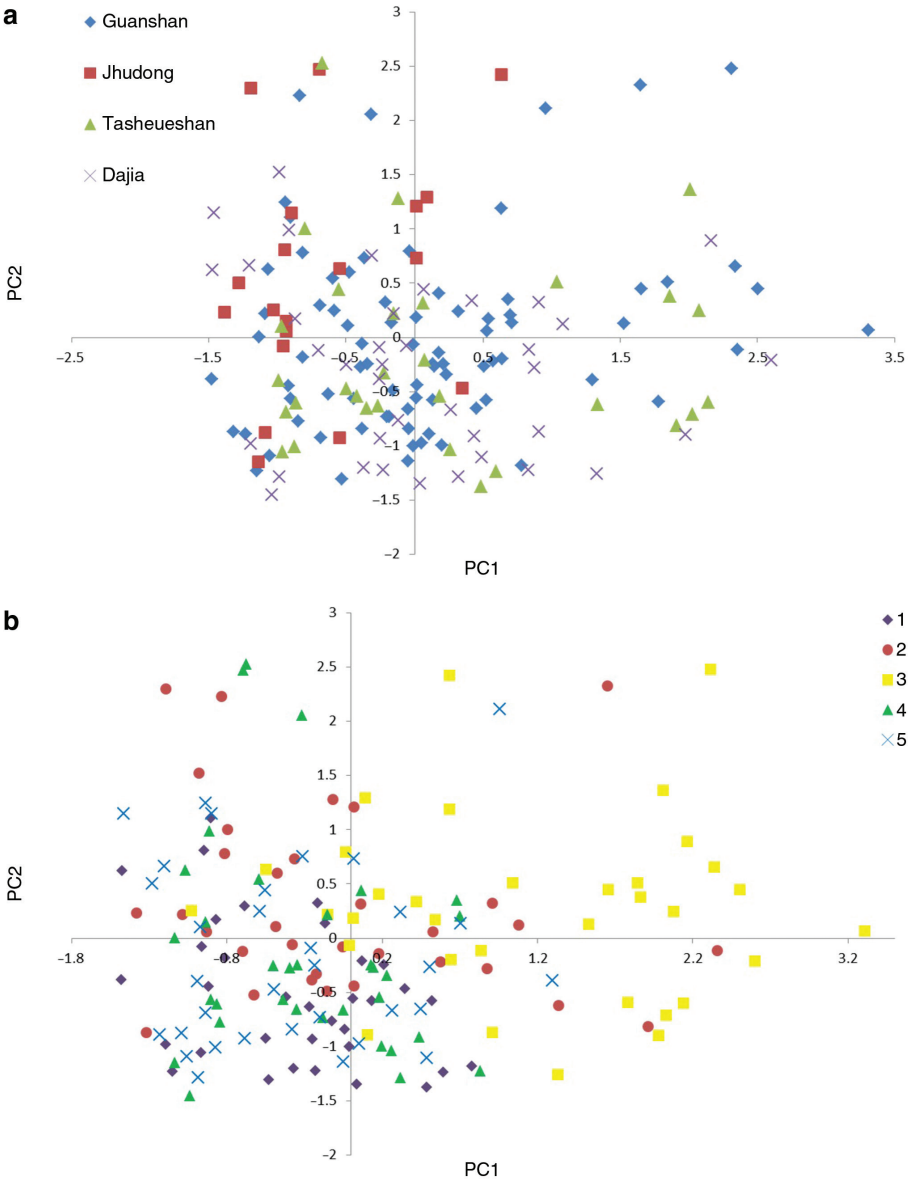

Figure 6: PCA score plots based on lignan quantification of Taiwania outer hW.
(a) grouped by provenances; (b) grouped by experimental plots.

Table 2: Eigen values and loadings of PCA based on lignan quantification of Taiwania outer heartwood.

Parameters	PC1	PC2	PC3	PC4
Eigen values	1614119.1	105174.8	57005.9	24823.8
Percentage	88.8	5.8	3.1	1.4
Cum. percent.	88.8	94.6	97.7	99.1
Loadings				
Hinokinin	0.000	0.000	0.000	0.000
Savinin	0.000	0.000	0.000	1.000
Taiwanin A	0.000	0.000	0.000	0.000
Taiwanin C	0.403	0.000	0.915	0.000
Taiwanin E	0.915	0.000	−0.403	0.000
Helioxanthin	0.000	1.000	0.000	0.000

PCA, Principal component analysis.

to taiwanin E; 5, 16, 28, 29, 50, 52 and 67 to 70 to helioxanthin (Table 3, Figure 7). Of these 38 lignan-associated integrals, 15 integrals had a $H^2 > 0.1$ (Table 3, Figure 7). For the 15 integrals with $H^2 > 0.1$, four integrals were able to unambiguously distinguish a specific lignan (14, 22 and 23 for savinin; 52 for helioxanthin), while the remaining 11 integrals contained proton signals of multiple lignans (Table 3). The correlation analysis between ^{1}H-NMR integrals 14, 22, 23 and 52 and the content of the six lignans determined by LC-MS displayed moderate but significant correlations for savinin and helioxanthin, but not the other four lignans (Table 4). Accordingly, the lignans-related ^{1}H-NMR-integrated signals have a high H^2. The high H^2 results were further confirmed by LC-MS, which also showed high H^2 values for the contents of hinokinin, savinin, taiwanin A, taiwanin C, taiwanin E and helioxanthin, with H^2 values of 0.82, 0.57, 0.52, 0.62, 0.73 and 0.79, respectively (Table 5).

The H^2 values obtained from the LC-MS are larger than the ones obtained from the ^{1}H-NMR. Probably, the ^{1}H-NMR signals are not specific enough to a compound because of the similar NMR spectra of the lignans in question. The analysis of the complete LC-MS data analyzed by PCA and the loading plot indicated that 315 and 361 *m/z* are the two most distanced outliers (Figure 8). The 315 and 361 *m/z* were contributed by the secondary metabolites with molecular weights (MW) of 314 and 360, which could be taiwania-quinone H and taiwania-quinone E, respectively (Chang et al. 2005). Both the taiwania-quinones are diterpenoids and have proton signals between δ8.00 and 6.00 ppm (integral 1–51), i.e. in the region that overlaps with that of the lignans.

The PCA results gives hints that the environmental factor, as implicated by the experimental plots, might contribute to some extent to abundance variation. According to the GLM analysis, the effect of the experimental plots is significant for each lignan determined by LC-MS (Table 5). Unlike PCA, which summarizes the data variation by the eigenvector with combinatorial effects of the variables, the GLM and LMM focus on a specific trait and analyze the contribution of the independent variables to the overall variance, which permits the more precise evaluation of the source variations. Again, the environmental effect plays a minor to moderate role concerning the lignan content. The abundance of lignans are genetically controlled and the lignan composition is highly heritable as indicated by the high H^2 values of both ^{1}H-NMR and LC-MS analyses.

Compared to the H^2 of *Pseudo. menziesii* metabolites (Robinson et al. 2007), *Pi. elliottii* (Lai et al. 2017) and *Pinus taeda* (Eckert et al. 2012), the heritability of the lignan content in Taiwania is in general higher. The interpretation of this finding is difficult because the genetic effects can be additive (gene dosage of multiple loci) and non-additive (dominance and epistasis). The heritability of *Pseudo. menziesii*, *Pi. elliottii* and *Pi. taeda* is relayed mainly on the additive effects. The high H^2 predicts that the lignan content as a trait in Taiwania should respond well to selection in breeding. The finding that the variance

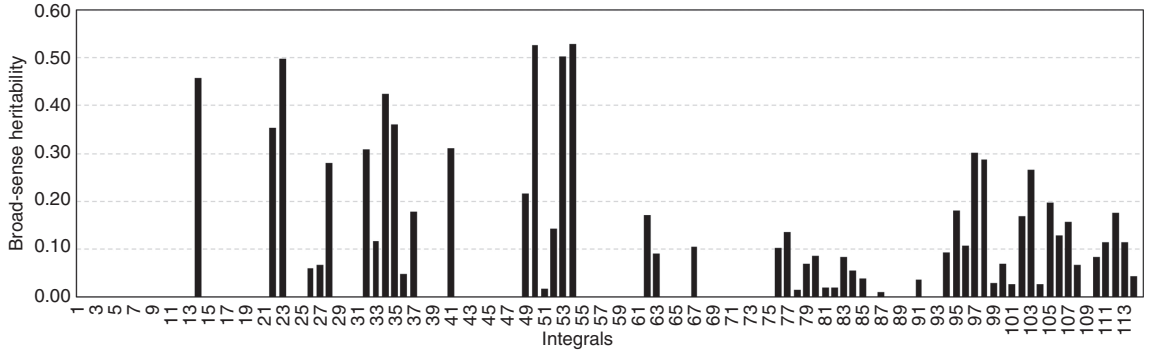

Figure 7: Broad-sense heritability (H^2) of ^{1}H-NMR spectral integrals.

Table 3: The corresponding positions[a] of protons on the lignans detected by ^{1}H-NMR in the outer heartwood of Taiwania.

Integral	Hinokinin	Savinin	Taiwanin A	Taiwanin C	Taiwanin E	Helioxanthin	H^2
4				C7			0
5						C5	0
10					C2		0
12			C7′				0
14		C7					0.46
16						C6	0
18				C2			0
22		C6					0.36
23		C2					0.50
26				C5			0.06
27				C6′	C6′		0.06
28		C5			C6′, C5	C2′, C5′, C6′	0.28
29						C2′, C5′, C6′	0
31				C2′, C5′			0
32			C6′	C5′	C2′, C5′		0.30
33	C6, C6′	C6′			C2′, C5′		0.12
34	C6, C6′	C2′, C5′	C7				0.42
35	C2, C2′						0.35
36	C2, C2′		C5′				0.05
37	C5, C5′		C5′				0.18
38	C5, C5′						0
39			C5, C6				0
43			C2′				0
47			C2				0
48				C20			0
49				C19	C19		0.22
50		C19			C20	C19	0.52
52						C20	0.13
53	C19, C20	C20					0.50
54		C20	C20				0.53
55			C20				0
56			C19				0
65				C9			0
67					C9	C9	0.10
68						C9	0
69						C9	0
70						C9	0
75			C9				0

[a]See Figure 1 for proton position.

^{1}H-NMR, proton nuclear magnetic resonance; H^2, broad-sense heritability.

Table 4: Correlation analysis between ^{1}H-NMR integrals 14, 22, 23 and 52 and the content of the six lignans determined by LC-MS.

Integral	Hinokinin	Savinin	Taiwanin A	Taiwanin C	Taiwanin E	Helioxanthin
14	0.010	0.285	−0.064	0.095	−0.029	−0.120
	(0.896)	(0.0002)	(0.408)	(0.219)	(0.704)	0.128
22	0.032	0.166	−0.065	0.023	−0.084	−0.117
	(0.678)	(0.031)	(0.401)	(0.770)	(0.278)	(0.137)
23	−0.005	0.247	−0.131	0.087	−0.037	−0.131
	(0.945)	(0.0012)	(0.083)	(0.262)	(0.632)	(0.096)
52	−0.036	−0.112	−0.045	−0.040	0.004	0.348
	(0.642)	(0.147)	(0.555)	(0.603)	(0.959)	(<0.0001)

Pearson's correlation coefficient (P-value).

^{1}H-NMR, proton nuclear magnetic resonance; LC-MS, liquid chromatography-mass spectrometry.

Table 5: GLM analysis broad-sense heritability of lignan abundances in the outer heartwood of Taiwania determined by LC-MS.

Lignan	P-value			V_G	V_E	H^2
	G	P	E			
Hinokinin	$<1\times10^{-4}$	0.098	0.21	71364.00	15571.91	0.82
Savinin	0.013	0.0034	$<1\times10^{-4}$	47716.00	36431.00	0.57
Taiwanin A	0.017	0.24	$<1\times10^{-4}$	1.67	1.57	0.52
Taiwanin C	0.0013	0.052	$<1\times10^{-4}$	737182.00	455651.20	0.62
Taiwanin E	$<1\times10^{-4}$	0.087	$<1\times10$	4332680.00	1653641.20	0.73
Helioxanthin	$<1\times10^{-4}$	$<1\times10^{-4}$	0.003	415176.00	108869.80	0.79

G, genotype effect; P, provenance effect; E, plot effect; V_G, genetic variance; V_E, environmental variance; H^2, broad-sense heritability; GLM, general linear model; LC-MS, liquid chromatography-mass spectrometry.

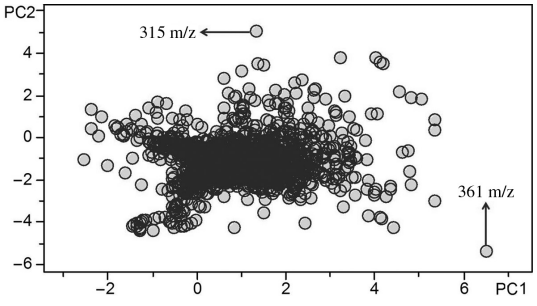

Figure 8: PCA loading plot based on the LC-MS metabolic profiles.

of the lignan content has mainly a genetic background leads to the conclusion that lignan contents can be substantially improved genetically.

Conclusions

In this study, ^{1}H-NMR spectroscopy and LC-MS were applied to analyze the metabolites in the hW of Taiwania. The LMM analysis showed that the integrals of the ^{1}H-NMR profiling for the lignans had a lower H^2 (from 0.11 to 0.45) than those based on LC-MS (from 0.52 to 0.82). Accordingly, LC-MS quantification was more robust and better suited for the estimation of a specific metabolite. Therefore, ^{1}H-NMR should be used mainly for high throughput metabolite predictions and semi-quantification. The LC-MS results showed that the six lignan contents had high H^2, which strongly suggested that the ^{1}H-NMR integrals of higher H^2 were contributed by the lignans (hinokinin, savinin, taiwanin A, taiwanin C, helioxanthin and taiwanin E). The genetic component is the dominant part of the abundance variation of the major lignans. The H^2 obtained in this study is an important parameter that can be applied to improve specific lignan contents genetically through the selection of elite genotypes.

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