



Natural variations in flavonoids and triterpenoids of *Cyclocarya paliurus* leaves

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Abstract *Cyclocarya paliurus* is widely distributed in subtropical areas of China. Secondary metabolites in the leaves, including flavonoids and triterpenoids, provide protection against diseases such as hyperlipidemia, diabetes, hypertension, and hyp immunity. In this study, 343 *C. paliurus* leaves were collected from 32 distinct populations covering most of the main distribution, to deduce the response of flavonoids and triterpenoids to seven environmental factors. Principal component analysis was performed using seven flavonoid and six triterpenoid compounds. The results show that altitude, latitude, and longitude significantly contributed to variations in total flavonoid and triterpenoid contents. In addition, the interaction of these metabolites was extensively correlated with environment changes, and therefore should be considered during selection and cultivation.

Keywords *Cyclocarya paliurus* · Environmental factors · Flavonoids · Secondary metabolite variation · Triterpenoids

Introduction

Cyclocarya paliurus (Batal) Iljnsk. is distributed across subtropical areas of China. The leaves have long been used to make a traditional sweet tea (Shu et al. 1995a, b; Fang et al. 2006). This species was investigated as a substitute to the common tea from *Camellia sinensis* (L.) Kuntze and was found to be beneficial to human health (Li et al. 2017a, b). The leaves have high concentrations of secondary metabolites that may be protective against a range of diseases, including hyperlipidemia, diabetes, and hypertension (Kurihara et al. 2003; Xie et al. 2006; Wang et al. 2017a; Li et al. 2017a, b). Previously, six triterpene compounds were isolated from the leaves by HPLC (high performance liquid chromatography): arjunolic acid, cyclocaric acid B, pterocaryoside B, pterocaryoside A, hederagenin, and oleanolic acid (Shu et al. 1995a, b; Wright et al. 2014; Shang et al. 2015; Cao et al. 2017; Liu et al. 2018). Seven flavonoid compounds have also been isolated, including quercetin-3-O-glucuronide, quercetin-3-O-galactoside, isoquercitrin, kaempferol-3-O-glucuronide, kaempferol 3-O-glucoside, quercetin-3-O-rhamnoside, and kaempferol-3-O-rhamnoside (Shang et al. 2015; Xie et al. 2015; Cao et al. 2017, 2018; Liu et al. 2018). Many studies have examined flavonoids and triterpenoids because of their health-promoting functions (Shu et al. 1995a, b; Xie et al. 2006, 2015; Li et al. 2017a, b).

Secondary metabolite biosynthesis in leaves are influenced by intrinsic and extrinsic factors (Szakiel et al. 2011). Intrinsic factors include genetic, genotypic and physiological status such as species, provenances, populations, families, plant growth stages and carbon–nitrogen metabolism.

Caowen Sun and Xulan Shang have contributed equally to this work.

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Extrinsic factors include biotic and abiotic environmental stimuli, such as reacting to pathogens, parasites, insects, animals (Szakiel et al. 2011), and light, soil carbon and temperature (Barnes et al. 2015). Generally, geographical variation research on secondary metabolites focuses on environmental factors along with genotype variations. These research programs select the main effects at work in a geographical area from comprehensive factors (Fang et al. 2011; Li et al. 2017a, b; Cao et al. 2018; Deng et al. 2018, 2019a, b). As a result, breeding and cultivation research is based on this information.

Considerable efforts are currently focused on genotypic and environmental interactions of *C. paliurus* germplasm secondary metabolite variation (Fang et al. 2006, 2011, Fang and Fu 2007; Deng et al. 2012, 2014, 2017; Li et al. 2017a, b). The research has made progress at the population level. Liu et al. (2018) found that geographical location significantly affects the phenolic composition of *C. paliurus*, and Zhou et al. (2019) noted that the content of total flavonoids and total polyphenols from 21 natural populations varied significantly. Deng et al. (2019a, b) reported that individual triterpenoids were primarily influenced by genotype, while the environment accounted for variations in total triterpenoid content. Cao et al. (2018) determined that, at the family level, flavonoid content variations of *C. paliurus* were significant. This has made it important to study secondary metabolite variations based on geography at the family level. More research has focused on variations of phenols, terpenoids and other secondary metabolites separately (Fang et al. 2011; Deng et al. 2012, 2014, 2015; Liu et al. 2018; Cao et al. 2018); limited information is available on the competing processes of flavonoid and triterpenoid accumulations. This has made it difficult to select beneficial plant traits and has impeded breeding and cultivation. Additional research on the geographical variations of *C. paliurus* secondary metabolites is therefore required. In this study, secondary metabolite variations in *C. paliurus* germplasm was

investigated over a wide distribution area. The objectives were to analyze flavonoid and triterpenoid variation patterns in leaves of naturally occurring plants at the family level, and to investigate relationships between secondary metabolites and environmental factors. The results will be important for targeting directed breeding approaches and for the identification of suitable areas.

Materials and methods

Plant material

Leaves of *C. paliurus* were collected from a total of 343 trees at 32 distinct localities covering most of the *C. paliurus* range in China in 2014. Samples were collected from 12 provinces, Anhui, Zhejiang, Fujian, Shanxi, Henan, Hunan, Hubei, Jiangxi, Guangxi, Guizhou, and Sichuan (Fig. 1), across a wide area of southern China at 9.0° latitude, 17.4° longitude, and 1370 m a.s.l. (Table 1). Only mature leaves were collected in October to ensure that secondary metabolites were fully developed. The trees were mainly dominant or subdominant greater than 20 years old. Samples were dried, crushed, and stored at room temperature prior to analysis (chemical analysis was carried out in 2014).

Flavonoid and triterpenoid determination

Approximately 75 g samples from each location were extracted with 80% ethanol before being incubated in a water bath at 90 °C for 2 h. This was followed by shaking for 15 min and centrifugation at 8000 r/min for 15 min. Supernatants were concentrated at 40 °C to obtain the extracts.

Flavonoid and triterpenoid contents were analyzed using high-performance liquid chromatography (HPLC) (Liu et al. 2018). Extracts were filtered through a 0.4- μ m polytetrafluoroethylene filter before HPLC analysis (Agilent 1200

Fig. 1 Sampling locations of *C. paliurus*

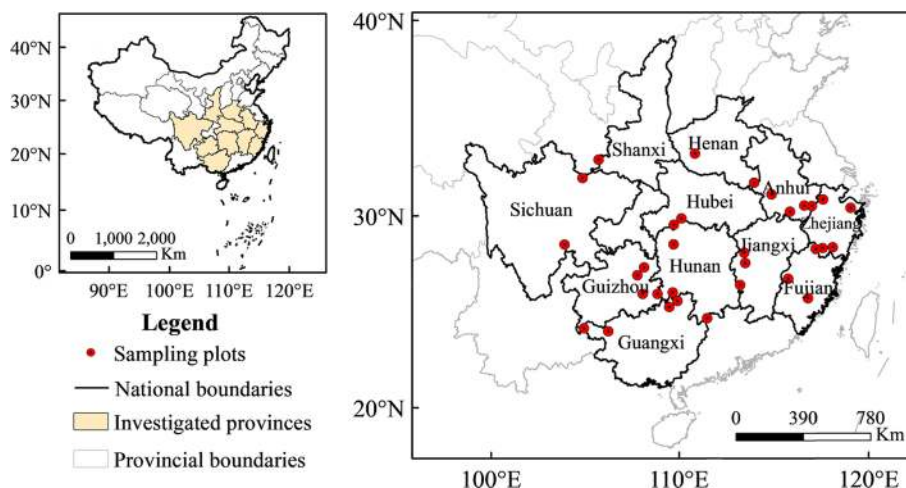


Table 1 Collection locations and associated environmental factors

Location	Latitude (°N)	Longitude (°E)	Altitude (m)	Annual mean temperature (°C)	Annual mean rainfall (mm)	Annual sunlight (h)	Frost-free days (day)	Sample size
Jixi County, Anhui	30.15	118.89	730	12.1	1726	1920	233	11
Jingde County, Anhui	30.23	118.45	610	13.8	1657	1784.1	240	3
Qimen County, Anhui	30.02	117.53	400	13.9	1775	1669	232	5
Shucheng County, Anhui	31.02	116.54	770	12.3	1606	1969	224	12
Ningbo City, Zhejiang	29.76	121.22	715	14	1527	1850	232	8
Longquan City, Zhejiang	27.91	119.19	1200	11.8	2119	1849.8	263	7
Wencheng County, Zhejiang	27.88	119.79	915	13.9	1913	1764.4	285	5
Anji County, Zhejiang	30.41	119.64	540	13.4	1472	1666	231	7
Pucheng County, Fujian	27.93	118.76	930	15.8	1998	1900	254	10
Quanzhou City, Fujian	25.43	117.93	500	18.1	1593	1908	320	3
Mingxi County, Fujian	26.57	116.93	564	17.4	1821	1788.6	261	12
Lueyang County, Shanxi	33.36	105.87	1200	13	714	1558.3	236	9
Nanzhao County, Henan	33.48	112	880	11.5	834	1850.5	219	8
Shangcheng County, Henan	31.72	115.55	920	12.2	1368	1763.1	222	6
Yongshun County, Hunan1	28.88	110.33	670	14.8	1492	1306	286	9
Shaoyang City, Hunan	26.37	110.13	1000	16.7	1320	1348.9	304	16
Yongshun County, Hunan2	24.92	112.03	845	17.4	1502	1758	308	10
Hefeng County, Hubei	29.88	110.42	1125	11.3	1499	1342	245	6
Wufeng County, Hubei	30.19	110.9	969	12.6	1650	1533	240	15
Xiushui County, Jiangxi	28.16	114.52	827	13.5	1655	1600.4	247	15
Jinggangshan County, Jiangxi	26.51	114.1	967	13.2	1816	1511	241	10
Fenyi County, Jiangxi	27.63	114.53	565	15.3	1691	1251	270	14
Baise City, Guangxi1	24.46	106.34	1448	15.6	1364	1906.6	357	12
Baise City, Guangxi2	24.61	104.95	1770	16.7	1212	1569.3	357	11
Longsheng County, Guangxi	25.62	109.89	606	14.5	1629	1244	314	11
Ziyuan County, Guangxi	25.92	110.38	850	15.5	1580	1275	300	10
Liping County, Guizhou	26.34	109.24	727	16.1	1311	1317.9	277	11
Jianhe County, Guizhou	26.37	108.38	1240	15.1	1265	1236.3	300	15
Shiqian County, Guizhou	27.35	108.11	1239	13.3	1256	1232.9	316	16
Yinjiang County, Guizhou	27.74	108.51	1032	14.5	1231	1255	300	7
Muchuan County, Sichuan	28.97	103.78	1200	17.3	1332	968	332	34
Guangyuan City, Sichuan	32.42	104.86	1570	14.1	1027	1292	243	12

series HPLC system, Waldbronn, Germany). A Waters 2489 ultraviolet detector (Waters, Milford, MA, USA) and an X-Bridge C18 column (250×4.6 mm) were used. Eluent A was water containing 0.01% formic acid while eluent B was acetonitrile with 0.01% formic acid. The ratio of A to B in stepwise elution was: 0–13 min, 92:8 (v/v); 13–28 min, 81:19; 28–42 min, 79:21; 42–60 min, 50:50; 60–64 min, 55:45; 64–74 min, 44:56; 74–90 min, 34:66; 90–95 min, 15:85; and 95–100 min, 0:100 (Liu et al. 2018). The flow rate was 1.0 ml/min, the injection volume 10 µl, the column

temperature 45 °C, and the wavelength 205 nm. The concentrations of individual flavonoids and triterpenoids were determined by comparison with external standards. Total flavonoids and total triterpenoids were the sum of individuals.

Environmental factors

Site data were collected from the collection sites with a GPS (JUNO@SCSD, Trimble, Sunnyvale, CA, USA), including longitude, latitude, and altitude. Annual mean

temperatures, rainfall, sunlight, and number of frost-free days were obtained from the nearest meteorological station or from the National Meteorological Data Service Centre of China (https://data.cma.cn/data/detail/dataCode/SURF_CLI_CHN_MUL_DAY_CES_V3.0.html) that provides the closest approximated values for each region.

Statistical analyses

Means and standard deviations (SD) were calculated for each flavonoid and triterpenoid content. Correlations analyses between environmental variables and flavonoid and triterpenoid contents were performed separately. The analyses were conducted using SPSS statistics software 19.0. Initial principal component analyses (PCAs) were carried out using flavonoid and triterpenoid contents as the variable factors to describe data variation characteristics. Additional PCAs were undertaken to examine the relationship between high flavonoid individuals, high triterpenoid individuals, and environmental factors. According to the PCA analyses and correlation analyses, the environment factors associated with secondary metabolite variations were selected, described by scatter plots, and tested by paired t-tests. All PCA analyses were conducted in the R v. 3.4.4 statistical environment (R Development Core Team 2018) with the package *vegan* v. 1.8–5 (Oksanen et al. 2007). Scatter plots and paired t-tests were conducted using the SPSS statistics software 19.0.

Results

Geographic distribution

A total of 343 *C. paliurus* accessions from 32 distinct populations were found across the *C. paliurus* distribution range in southern China (Fig. 1; Table 1). The geographic distribution ranged from Henan province to Guangxi province, and from Zhejiang province to Guizhou province, showing that *C. paliurus* is widely adapted. However, there are preferred

growth features and habitats: (1) dominant or subdominant in competition; (2) growing in valleys or on limestone hills with an altitude limit of 400–1770 m; and, (3) scattered in relatively small populations in mixed forest.

The altitude of *C. paliurus* ranged from 400 m a.s.l. (Anhui) to 1770 m a.s.l. (Guangxi). Mean annual rainfall ranged from 714 mm (Shanxi) to 2119 mm (Zhejiang), and annual mean temperatures from 11.3 °C (Hubei) to 18.1 °C (Fujian). Annual hours of sunlight ranged from 968 h (Sichuan) to 1969 h (Anhui), while the number of frost-free days ranged from 219 d (Henan) to 357 d (Guangxi).

Secondary metabolites in *C. paliurus* leaves

This study primarily focused on flavonoid and triterpenoid variations and chemical traits identified by HPLC according to Cao et al. (2017), including quercetin-3-O-glucuronide, quercetin-3-O-galactoside, isoquercitrin, kaempferol-3-O-glucuronide, kaempferol 3-O-glucoside, quercetin-3-O-rhamnoside, kaempferol-3-O-rhamnoside, arjunolic acid, cyclocaric acid B, pterocaryoside B, pterocaryoside A, hederagenin, and oleanolic acid.

To analyze their variations, the maximum, minimum, and average amounts of flavonoids and triterpenoids were calculated together with their respective standard deviations (Table 2). These findings indicate that most flavonoid and triterpenoid contents varied greatly. Overall, triterpenoids (1.7–44.5 mg/g) showed relatively higher variation than flavonoids (0.6–27.1 mg/g). Among the flavonoids, quercetin-3-O-glucuronide (0–6.0 mg/g) and kaempferol-3-O-glucuronide (0.1–6.9 mg/g) varied widely, with the means being 1.8 mg/g and 1.4 mg/g, respectively. Of the triterpenoids, arjunolic acid (0.2–11.5 mg/g), pterocaryoside B (0–19.2 mg/g), and pterocaryoside A (0.1–16.4 mg/g) showed significantly wide variations with average values of 3.4 mg/g, 2.7 mg/g and 3.6 mg/g, respectively. One interesting result is that the relative CV (coefficient of variation) for isoquercitrin (1.4) and kaempferol 3-O-glucoside (1.4) are much higher than for the other flavonoids and triterpenoids.

Table 2 Flavonoid and triterpenoid compositions (mg/g) in 343 *C. paliurus* individual leaves

Flavonoids	Min	Max	Mean	SD	CV	Triterpenoids	Min	Max	Mean	SD	CV
Quercetin-3-O-glucuronide	0.0	6.0	1.8	1.3	0.7	Arjunolic acid	0.2	11.5	3.4	2.0	0.6
Quercetin-3-O-galactoside	0.0	4.4	0.6	0.4	0.8	Cyclocaric acid B	0.3	6.4	1.5	1.1	0.7
Isoquercitrin	0.0	8.4	0.4	0.6	1.4	Pterocaryoside B	0.0	19.2	2.7	2.9	1.1
Kaempferol-3-O-glucuronide	0.1	6.9	1.4	0.9	0.6	Pterocaryoside A	0.1	16.4	3.6	2.7	0.8
Kaempferol 3-O-glucoside	0.0	4.0	0.4	0.6	1.4	Hederagenin	0.0	4.7	1.0	0.7	0.7
Quercetin-3-O-rhamnoside	0.0	1.2	0.2	0.2	0.7	Oleanolic acid	0.1	1.5	0.4	0.2	0.6
Kaempferol-3-O-rhamnoside	0.0	4.8	1.5	1.0	0.6	Total triterpenoid	1.7	44.5	12.6	7.0	0.6
Total flavonoid	0.6	27.1	6.3	3.4	0.5						

SD=standard deviation. Zero indicate that the compound concentration is lower than detection limit

Correlation analyses

As shown in Table 3, correlation analyses showed flavonoids and triterpenoids with limited correlation to three geographical and three climate factors. Of these, latitude was negatively correlated with arjunolic acid ($R = -0.368, P < 0.05$) content while longitude was negatively correlated with isoquercitrin levels ($R = -0.374, P < 0.05$). Altitude was negatively correlated with total triterpenoid levels ($R = -0.364, P < 0.05$) and pterocaryoside A content ($R = -0.360, P < 0.05$). For climate factors, average annual temperatures were negatively correlated with kaempferol-3-O-glucuronide ($R = -0.43, P < 0.05$); annual precipitation was positively correlated with pterocaryoside A ($R = 0.390, P < 0.05$) and negatively with isoquercitrin ($R = -0.384, P < 0.05$) and kaempferol-3-O-glucoside ($R = -0.426, P < 0.05$). The number of annual frost-free days was negatively correlated with hederagenin ($R = -0.391, P < 0.05$).

Based on the analyses, there were moderate correlations at $P < 0.05$ significance level and the maximum absolute value of the correlation coefficient was weak at 0.426. Overall, the correlation between *C. paliurus* leaf flavonoids and triterpenoids and environmental factors appears to be very limited. While considering correlation analyses at a macro-scale might lead to the loss of information, a deeper analysis of individual flavonoid and triterpenoid variations was required.

Principal component analysis (PCA)

A PCA biplot analyzed the data of *C. paliurus* leaf flavonoids and triterpenoids (Fig. 2). The first two PCAs, analyzed from 15 variables of flavonoids and triterpenoids, explained 55.0% of the total variation (PC 1 explained 31.4% and PC 2 explained 23.6%). Most flavonoid and triterpenoid

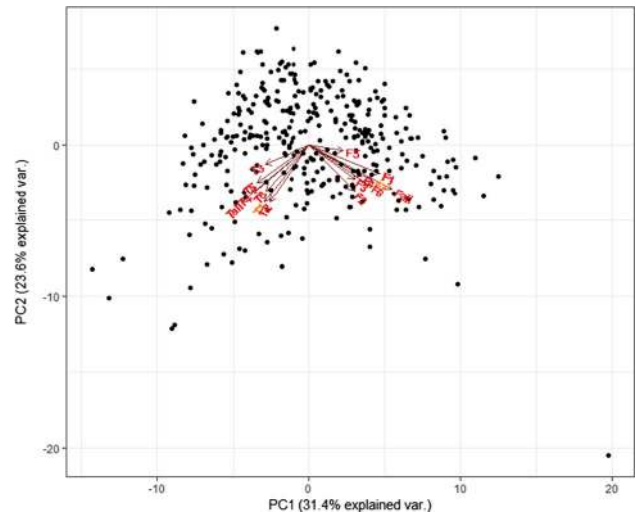


Fig. 2 Principal component analysis of *C. paliurus* flavonoids and triterpenoids; T1: arjunolic acid, T2: cyclocaric acid B, T3: pterocaryoside B, T4: pterocaryoside A, T5: hederagenin, T6: oleanolic acid, Tall: total triterpenoid, F1: quercetin-3-O-glucuronide, F2: quercetin-3-O-galactoside, F3: isoquercitrin, F4: kaempferol-3-O-glucuronide, F5: kaempferol 3-O-glucoside, F6: quercetin-3-O-rhamnoside, F7: kaempferol-3-O-rhamnoside, and Fall: total flavonoid

Table 3 Correlation analysis results between environmental factors and leaf traits

	Lat	Lon	Alt	Te	Prec	Sundur	Ff
Arjunolic acid	-0.368*	0.095	-0.335	0.231	0.287	0.032	0.125
Cyclocaric acid B	-0.277	0.146	-0.275	0.199	0.249	0.021	0.118
Pterocaryoside B	-0.203	0.121	-0.224	-0.035	0.211	-0.029	-0.06
Pterocaryoside A	-0.208	0.296	-0.360*	0.042	0.390*	-0.004	-0.132
Hederagenin	0.269	0.345	-0.206	-0.186	0.044	0.253	-0.391*
Oleanolic acid	0.164	0.317	-0.236	-0.06	0.121	0.102	-0.309
Total triterpenoid	-0.258	0.235	-0.364*	0.076	0.336	0.023	-0.063
Quercetin-3-O-glucuronide	0.183	-0.005	0.085	-0.346	0.095	0.012	-0.068
Quercetin-3-O-galactoside	-0.071	0.034	-0.112	0.183	-0.04	0.149	0.084
Isoquercitrin	0.169	-0.374*	0.233	0.07	-0.384*	-0.084	0.071
Kaempferol-3-O-glucuronide	0.319	-0.114	0.195	-0.403*	-0.076	-0.109	-0.18
Kaempferol 3-O-glucoside	0.284	-0.285	0.226	0.103	-0.426*	-0.032	-0.011
Quercetin-3-O-rhamnoside	0.156	-0.144	0.068	-0.124	-0.04	-0.178	-0.033
Kaempferol-3-O-rhamnoside	0.088	-0.263	0.103	-0.021	-0.073	-0.197	0.163
Total flavonoid	0.282	-0.232	0.192	-0.21	-0.164	-0.089	-0.013

*Significant correlation at the $P < 0.05$ level

Alt: altitude, Te: annual average temperature, Prec: average annual precipitation, Lat: latitude, Lon: longitude, Sundur: sunshine duration, and Ff: frost-free days

information was included, especially for total triterpenoid (0.99) and total flavonoid (0.98) content (Table 4). Information for cyclocaric acid B (0.77), pterocaryoside B (0.89), pterocaryoside A (0.82), quercetin-3-O-glucuronide (0.82), Isoquercitrin (0.78), and kaempferol-3-O-glucoside (0.75) was also included. Based on the results, two principal component analysis adequately described the data structure of *C. paliurus* leaf flavonoids and triterpenoids.

As shown in Fig. 2, different types of flavonoids showed almost the same direction of variation, indicating that they changed in basically the same pattern, as did the triterpenoids. However, flavonoids and triterpenoids showed different directions in variation but it appeared that they interacted to some extent. According to Figs. 2 and 3, the resources could be divided into three groups: group 1 with high flavonoid and low triterpenoid contents; group 2 with high triterpenoid and low flavonoid contents; and, group 3 with low flavonoid and low triterpenoid contents. In other words, flavonoids and triterpenoids suppressed each other when either levels increased. With the total flavonoid content being more than 10 mg/g, 45 high flavonoid (HFla) individuals were divided, including 12 from Sichuan, 10 from Hubei, 5 from Anhui, 4 from Shanxi, 3 from Jiangxi, 4 from Guangxi, one from Guizhou, 4 from Zhejiang, and one from Fujian. Twenty high triterpenoid (HTri) individuals (levels more than 25 mg/g) were divided, including 12 from Jiangxi, 4 from Hunan, and one each from Henan, Anhui, Guizhou and Guangxi.

To determine the extent to which environmental factors affect secondary metabolites, additional PCAs were carried out (Fig. 4). The first two, generated from six environmental variables, including latitude, longitude, altitude, annual average temperatures, sunshine duration, and annual frost-free days explained 74.9% of all variations. The PCA model extracted most of the environmental factor information of HFla, HTri, and normal

Table 4 Paired t-tests of environmental factors according to secondary metabolite contents

Pairs	Environmental factors	No.	Correlation coefficient	P value
HTri-LTri	Altitude	32	0.399	0.024*
HFla-LFla	Latitude	32	-0.304	0.091
HFla-LFLa	longitude	32	0.307	0.087
HFla-HTri	longitude	25	0.357	0.079
HFla-HTri	Altitude	25	-0.514	0.009**
HFla-HTri	Rainfall	25	0.416	0.039*

*Significant correlation at the $P < 0.05$ level, **Extremely significant correlation at the $P < 0.01$ level

HTri: locations with high triterpenoid individuals, LTri: locations with low triterpenoid individuals, HFla: locations with high flavonoid individuals, LFla: locations with low flavonoid individuals

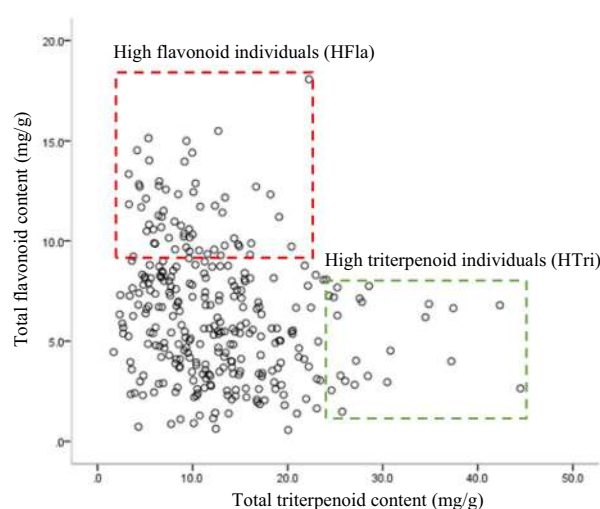


Fig. 3 Analysis of *C. paliurus* germplasm flavonoids and triterpenoids (mg/g)

individuals. Sites with more HFla individuals (Fig. 4) were mostly distributed in higher latitudes or at higher altitudes, while sites with more HTri individuals were mostly distributed in areas with higher rainfall. This confirms that environmental factors play a role in secondary metabolite variation. When comparing the environmental factors

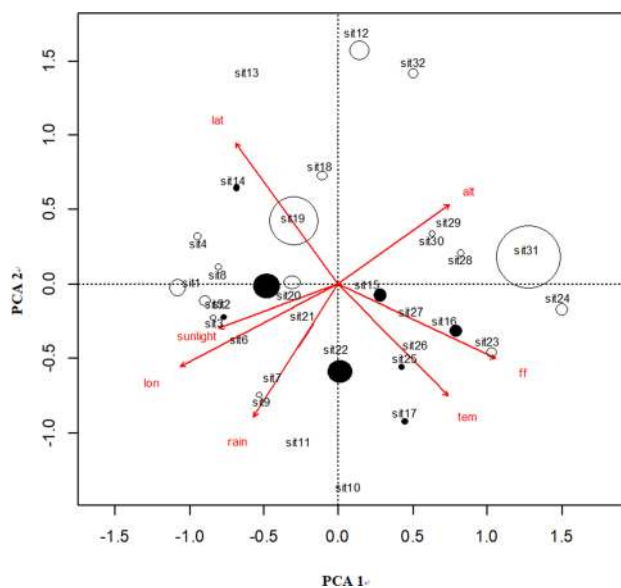


Fig. 4 Principal component analysis of environmental factors of *C. paliurus* germplasm location. lat: latitude, alt: altitude, lon: longitude, tem: annual average temperature, rain: average annual precipitation, sunlight: sunshine duration and ff: frost-free days; sit indicates population locations. Open circle indicate locations with high-flavonoid (HFla) individuals, the larger area the more individuals. Filled circle indicate locations with high-triterpenoid (HTri) individuals, the larger area the more individuals

associated with the distribution of HFla and HTri individuals together, the results were not totally symmetrical; there was an overlap that consisted of part of the scattering area of the HTri individuals and the whole of the scattering area of the HFla individuals (Fig. 4).

T-tests were conducted to identify any significant environmental differences between individuals with more secondary metabolites and normal individuals (Table 4). The analysis was carried out between environmental factors of HTri and LTri individuals, HFla and LFla individuals, and HTri and HFla individuals (Fig. 3). Notably, compared with HTri locations, HFla locations were significantly negatively correlated with altitude, with correlation coefficients as high as -0.514 . Moreover, rainfall was also significantly positively correlated between HTri locations and HFla locations at the $P < 0.05$ level, with the correlation coefficient being 0.416 .

From the PCA and t-test results, scatter plots were developed to examine the geographical variations in secondary metabolites more closely. As shown in Fig. 5a, HTri individuals were distributed with longitude, ranging from almost 108° E to 118° E, and across altitudes from sea level to 1000 m. However, HFla individuals were generally scattered in the northwest portion of the *C. paliurus* range (Fig. 5b). In addition, compared with HFla individuals (Fig. 5c), HTri individuals were scattered across lower altitudes from sea level to 1000 m, while HFla individuals were relatively insensitive to altitude (from sea level to 2000 m). Taken together, these results indicate that secondary metabolites were definitely affected by environmental factors, but flavonoid and triterpenoid synthesis were not completely opposed.

Discussion

Genetic and environmental factors and their interactions are known to affect plant secondary metabolite synthesis (Hamilton et al. 2001); the mechanism underlying this process requires further investigation. According to the carbon-nutrient balance hypothesis (CNBH), production of phytochemical defense substances is limited by the available carbon and nitrogen nutrients in plant tissues. Massad et al. (2012) found that flavonoids in *Pentaclethra macroloba* (Willd.) Kuntze seedling leaves fit the CNBH while triterpenoids did not. They attributed this to different biosynthetic pathways of the two secondary metabolites. Wang et al. (2017b) found that the triterpenoid saponin, and the total flavonoid content of *Glycyrrhiza uralensis* Fisch adventitious roots were synergistically enhanced significantly by the combination of methyl jasmonate and phenylalanine, indicating an interaction between flavonoid and triterpenoid synthesis. According to the analysis in this study, because of the different accumulating directions between flavonoids and triterpenoids, the *C. paliurus* leaves were divided into high triterpenoid, high flavonoid and normal plants, while no leaves with high contents of both were found. Alqahtani et al. (2015) considered that, in the herbaceous *Centella asiatica* (L.) Urban, flavonoid compounds and triterpenes showed similar patterns influenced by the time of collection. Our results suggest, however, that when stimulated by environment factors, only either flavonoids or triterpenoids increased but not both, contrary to Alqahtani et al. (2015) and Wang et al. (2017b). Interaction between flavonoids and triterpenoids in *C. paliurus* leaves may be associated with environmental conditions or trade-offs between growth and defense (Keinänen et al. 1999; Laitinen et al. 2005). From a

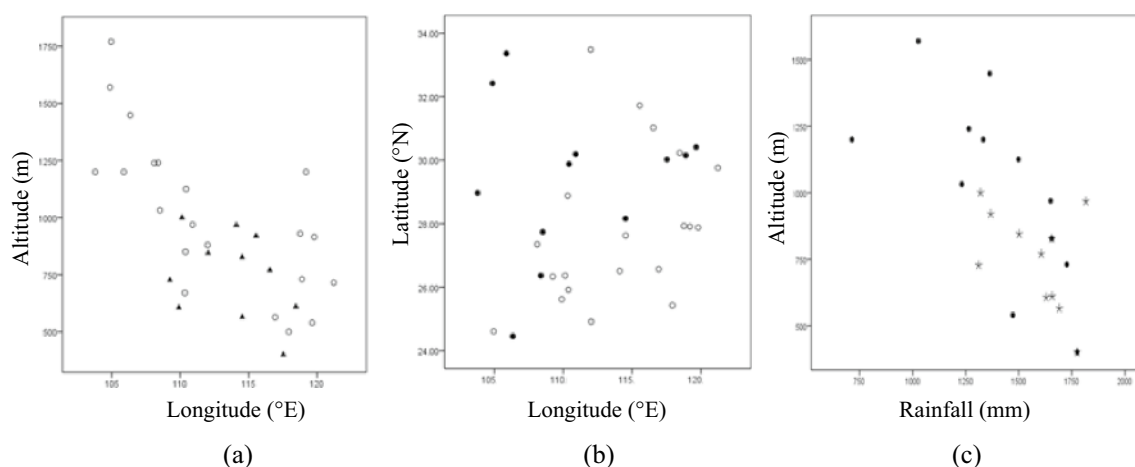


Fig. 5 Environment characteristics of high-triterpenoid (HTri) individuals and high-flavonoid (HFla) individuals; **a** Filled triangle indicate locations of high-triterpenoid (HTri) individuals and Open circle locations of other (LTri) individuals. **b** Filled circle indicate loca-

tions of high-flavonoid (HFla) individuals and Open circle locations of other (LFla) individuals. **c** Filled circle indicate locations of high-flavonoid (HFla) individuals and * indicate high-triterpenoid (HTri) individuals

commercial perspective, *C. paliurus* leaves should be studied in two directions, ones with high flavonoids and ones with high triterpenoid contents.

Previous studies have analyzed the responses of secondary metabolites to abiotic factors (Deng et al. 2015, 2018; Liu et al. 2018), and phenotypic variations in secondary metabolite accumulation (Fang et al. 2011; Cao et al. 2017, 2018) of *C. paliurus*. While almost all genotypic investigations have been carried out at the population level (Deng et al. 2015, 2017, 2019a, b; Liu et al. 2016), Cao et al. (2018) recognized this and considered that there were large variations between individuals in secondary metabolite contents. Our research is in agreement with Cao et al. (2018) and has analyzed to the individual level, in case there was information missed at the population level, including genotypic, microclimatic, and soil aspects.

According to previous findings, environmental stress often significantly influences flavonoid synthesis in plants, especially stress caused by UV radiation and low temperatures (Kootstra et al. 1994; Casati and Walbot 2003; Valkama et al. 2004; Stracke et al. 2010). Jaakola and Hohtola (2010) found that northern climates, possibly because of long days of sunlight and cool night temperatures, had a positive impact on flavonoid biosynthesis. This effect remained obvious, in spite of variations between species and within individual groups. In addition, Liu et al. (2016) found that the flavonoid contents of *C. paliurus* leaves were highly positively correlated with total solar radiation. Our results show that the northwestern part of the *C. paliurus* range at high latitudes or at higher altitudes contained almost all the high-flavonoid individuals. Compared with the study of Deng et al. (2015), no significant environmental factors were identified in specific ranges, while a general trend was found in the whole *C. paliurus* population. This is in agreement with Jaakola and Hohtola (2010) and suggests that areas with higher altitudes or latitudes provided sufficient solar radiation and cool night temperatures to stimulate flavonoid synthesis. This is also consistent with Liu et al. (2016) who found that *C. paliurus* leaves from Muchuan County had the highest flavonoid content. Our results suggest that high-flavonoid *C. paliurus* resources should be investigated from the northwest areas with higher altitudes.

Park et al. (2005) and Schlag and McIntosh (2006) found that the same species growing in different environments generally had differences in triterpenoid variety and content. Fan et al. (2013) and Sun et al. (2018) found that higher temperatures in the range of soapberry (*Sapindus mukorossi* Gaertn.) resulted in higher yields of the triterpenoid saponin. Jiao and Gao (2011) and Zeng et al. (2012) also reported that temperature significantly influenced the synthesis of the triterpenoid saponin. Most studies agree that triterpenoid synthesis is affected more significantly by environment than genotype. In *C. paliurus*

however Deng et al. (2018), found that individual triterpenoids could be influenced by genotype while the environment influenced the total triterpenoid variation. Our study included more sites and so allowed a comparison of the environmental factors between high triterpenoid plants and other individuals. This is in partial agreement with Deng et al. (2018), however, we found that altitude was significantly correlated with *C. paliurus* triterpenoid content with high triterpenoid individuals scattered almost solely on sites under 1000 m and below longitudes of 117° N. Based on Deng et al. (2018), this suggests that lower altitudes and rainfall might promote triterpenoid synthesis across the whole distribution area. It was also found that high triterpenoid individuals require relatively higher precipitation and lower altitudes, while high flavonoid plants are distributed across a broader range of conditions. The results agreed with a study of balsam pear that showed that higher relative humidity promotes total saponins and inhibits total flavonoids (Li et al. 2015). Higher temperatures and photosynthetically active radiation promote flavonoid accumulations but are unfavorable for saponin synthesis (Li et al. 2015).

We found that environmental factors influenced accumulation when the level of one kind of secondary metabolite was much higher than the other. However, this only occurred at the family level and not at the population level in each location, implying that potential genotypic or environmental aspects might be involved as indicated previously (Deng et al. 2015, 2017). This is in agreement with Deepak et al. (2018) who found that genotype-related secondary metabolite variations (between families or populations) were much stronger than provenance in silver birch. Genotypes, even between families, differ in their responses to the environment (Keinänen et al. 1999; Laitinen et al. 2005), so the combination of different genotypes and their environments may produce high secondary metabolite content. Tree age and vigor, the age of leaves, soil conditions, and microclimate might also play a role in this process. In this study, the key environmental factors and distribution correlated with high secondary metabolite accumulation were identified.

Conclusions

Environmental factors that affect flavonoid and triterpenoid levels were identified and this information will be valuable for the selection of suitable *C. paliurus* cultivation areas and directed breeding. Further research should examine interactions between genetic and environmental factors in secondary metabolite synthesis to help identify mechanisms underlying secondary metabolite variations of this economically important species.

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