Variation in Metabolic Characteristics of *Chrysanthemum indicum* from Different Habitats: Implications for cultivation and breeding

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Abstract

Chrysanthemi Indici Flos (CIF), the capitulum of *Chrysanthemum indicum*, is widely used as Chinese medicine, spice, and tea in many areas, its extracts are also frequently used in daily chemical products and cosmetics. Although CIF-related products are becoming more abundant because of its excellent biological activity, its metabolic characteristics and the accumulation of metabolites are still unclear. A total of 802 metabolites were identified in CIF by widely targeted metabolomics, and significant differences were found in the accumulation of metabolites of CIF from different habitats. Particularly, the differences in metabolite accumulation in CIF were significantly correlated with the geographical distribution in mountainous areas, but not with climate. Through multivariate statistical analysis, the seven samples from different habitats could be divided into four categories, and the significantly changed metabolites between different categories were mainly concentrated in the flavonoid synthesis pathway. Judging from the metabolic level, it was supported to separate the *Ch. nankingense* (Juhuanao) into a single species. In addition, the corresponding candidate biomarkers were screened in this study to identify the origin of CIF and distinguish the Juhuanao. It can be inferred that widely targeted metabolomics has great application potential in authenticity identification, origin tracing and quality evaluation of CIF. There were abundant metabolic differences among different populations of *Ch. indicum*, which provided abundant materials for the selection and breeding of fine varieties.

1. Introduction

Chrysanthemi Indici Flos (CIF) is the dried capitulum of *Chrysanthemum indicum* L., a commonly used traditional Chinese medicine (TCM), which has the following effects: clearing away heat, detoxifying, antibacterial and anti-inflammatory (Chinese Pharmacopoeia Commission 2020). Pharmacological studies have shown that CIF has many biological activities such as anti-apoptosis, scavenging oxygen free radicals, antibacterial and anti-inflammatory (Chen et al. 2008; Ding et al. 2016; Eun-Kyung 2009; Zhu et al. 2005). The Chinese Pharmacopoeia stipulated that linarin was the content determination index component of CIF. In addition, because of its rich essential oils and excellent bacteriostasis, the extract of CIF has been used in many personal care products, such as toothpaste, hand sanitizer, shower gel, facial cleanser, toilet water, whitening cream, etc. (Zhu et al. 2005; Song et al. 2020a). *Ch. indicum* is a polymorphic species with many ecological, geographical or eco-geographic populations. It is widely distributed in China as well as central and southern Japan (Lim 2014). There are many changes in the posture of *Ch. indicum*, which are mainly manifested in the diameter of the flower head, the density of the stems and branches, and the depth of the leaves. Among them, there is a diploid *Ch. indicum* endemic to Nanjing, named *Chrysanthemum nankingense* (Nakai) Tzvel (Chinese name Juhuanao), whose tender shoots can be used as a vegetable. Compared with other *Ch. indicum*, Juhuanao has a unique cool aroma, weak bitter taste and is usually not medicinal (Song et al. 2018). At present, the vast majority of CIF in the market comes from wild resources, but it shows the trend that the natural renewal speed of wild resources can not meet the market demand, resulting in the increase of its price year by year. However, the study of artificial cultivation has just started, and the work of breed selection is urgent to be solved. The

quality of medicinal plants is mainly determined by their chemical constituents. More and more studies have found that there is variability in the chemical constituents of TCMs from different places of origin and even among different individuals from the same place of origin (Wang et al. 2020; Sun et al. 2020). The degree of this variation is manifested as an order of magnitude difference in content and even the difference between presence and absence. Therefore, it is of great significance to detect the metabolites of medicinal herbs from different origins for the evaluation of their medicinal quality.

Metabolomics is a technique for the qualitative and quantitative analysis of all metabolites in the body of organisms. Since metabolites are the basis of phenotypes of organisms, the application of metabolomics techniques can help to understand biological processes and mechanisms more intuitively and effectively (Brennan 2017; Mallick et al. 2005; Kanu et al. 2007; Bose et al. 2015). Metabolomics provides a powerful means for the complex system research and new drug development of TCMs, and it has been widely used in the material basis research, quality control, efficacy mechanism, toxicity mechanism, safety evaluation and other fields of TCMs. Among them, widely targeted metabolomics detection technology is a new technology which integrates the advantages of non-targeted and targeted detection technologies. This technology has the advantages of high throughput, high sensitivity and wide coverage for relative quantitative detection of metabolites, and can accurately detect 500–1,000 primary and secondary metabolites simultaneously, providing an important basis for the analysis of metabolic pathways and biological mechanisms (Chen et al. 2013). In this study, on the basis of germplasm collection of *Ch. indicum* resources, representative types were screened for widely targeted metabolomics analysis to explore the differences in the metabolic levels of *Ch. indicum* (including Juhuanao) in different habitats. To further explore the factors affecting the quality of *Ch. indicum*, so as to provide guidance for breed selection and standardized cultivation.

2. Materials And Methods

2.1 Sample information

The experimental material of this study is CIF, which is made from the capitulum of the plant *Ch. indicum* after drying at 40°C. We collected CIF samples from seven typical regions, and the specific sampling locations are shown in Table 1. Among them, the CIF sample from Nanjing is a unique diploid plant Juhuanao. We set three 2-by-2-metre quadrats in each sample plot, collected the blooming capitulum of the *Ch. indicum* in each quadrat and mixing them for repetition.

Table 1
Sample information of *Ch. indicum* from different regions

Sample ID	Distribution area	East longitude	Northern latitude	Mountain range
CG	Chenggu County, Shaanxi Province	107°17'16"	32°59'35"	Qinling Mountains
SN	Shangnan County, Shaanxi Province	110°41'59"	33°33'57"	Qinling Mountains
HN	Huaining County, Anhui Province	116°56'24"	30°44'28"	Ta-pieh Mountains
MC	Macheng County, Hubei Province	115°04'36"	31°9'53"	Ta-pieh Mountains
PY	Pingyu County, Henan Province	114°49'23"	32°48'52"	Ta-pieh Mountains
PL	Pinglu County, Shanxi Province	111°02'21"	34°45'57"	Taihang Mountains
NJ(Juhuanao)	Nanjing, Jiangsu Province	118°58'46"	32°15'53"	plain

2.2 Determination of the total flavonoids and linarin

Rutin (CAS Number:153-18-4, purity $\geq 98\%$, Lot: Y19N7S25244, Yuanye Biological Co., Ltd., Shanghai, China) was used to calibrate the total flavonoid content using spectrophotometric detection, as described by Wang et al. (2019). The standard curve equation of rutin is $y = 0.9858x - 0.0745$, $R^2 = 0.9987$. High-performance liquid chromatography (HPLC, Agilent 1100 Compact LC G1316A) was used to detect the content of linarin according to the standard of Chinese Pharmacopoeia. The standard of linarin (CAS Number:480-36-4, purity $\geq 98.5\%$, Lot: 110753–201817) was from the National Institutes for Food and Drug Control.

2.3 Metabolomics detection

The instruments and methods used for the preparation of crude extract and the detection of the LC-ESI-MS/MS system were consistent with those described in the references (Wang et al. 2019). Based on the self-established Metware database and the public database of metabolite information, qualitative analysis was conducted according to the secondary spectral information. Metabolite quantification was completed by multiple reaction monitoring (MRM) analysis using triple quadrupole mass spectrometry (Fraga et al. 2010). It was worth noting that all samples were mixed into quality control (QC) samples in this study to verify the precision of the method. In addition, one quality control sample was inserted after every five samples to monitor the repeatability of the analysis process.

2.4 Data analysis

The relative contents of metabolites were normalized by the range method. The accumulation mode of metabolites among different samples is subjected to hierarchical cluster analysis (HCA), principal

component analysis (PCA), and correlation analysis through R software (www.r-project.org/). Based on the results of the orthogonal partial least squares discriminant analysis (OPLS-DA), significantly changed metabolites (SCM) were further screened by combining the obtained variable importance in projection (VIP) with the P-value or fold change of univariate analysis. The specific screening criteria were $VIP \geq 1$ and fold change ≥ 2 or fold change ≤ 0.5 . The SCM were corresponding to the KEGG (Kyoto Encyclopedia of Genes and Genomes, <https://www.kegg.jp/>) database for annotation through the R package, and then the enrichment analysis of the metabolic pathways of SCM were performed according to the annotation results.

The meteorological data of the habitats, including precipitation, temperature, wind speed and humidity, were obtained by the National Meteorological Information Center of China (<http://data.cma.cn/>). The clustering analysis of climate factors and metabolites of CIF from the seven habitats was carried out by SPSS Statistics, and the corresponding proximity matrices were obtained. Some key parameters in the clustering analysis were set as between-groups linkage and pearson correlation coefficient. The proximity matrices obtained above were imported into NTSYS-pc software for Mantel test to evaluate the correlation between climate factors and the accumulation of metabolites, in which the replacement test was set to 1 000 times.

3. Results

3.1 Analysis of the total flavonoids and linarin in CIF from different habitats

The total flavonoid content is a common test of TCMs, and linarin is the index component of CIF specified in the current Chinese Pharmacopoeia. Therefore, in this study, the contents of total flavonoids and linarin were detected in CIF collected from different habitats. The locations of *Ch. indicum* samples and the characteristics of its capitulum are shown in Fig. 1A and B. The results showed that the contents of total flavonoids in MC and HN samples were relatively high, while the PL sample exhibited the lowest content. In terms of the content of linarin, the HN, MC and PY samples were all higher than 2.5%, while the NJ sample was only 0.03%, and it was not detected in PL and CG (Fig. 1C).

3.2 Metabolite detection and quality control analysis of CIF

Based on the self-established Metware database, the qualitative detection of metabolites was carried out by MRM. A total of 802 metabolites were identified in CIF from seven different origins, which were divided into 22 categories, among which flavonoids (209, including flavones, flavonols, flavanones, isoflavones, anthocyanins, and proanthocyanidins), organic acids and derivatives (107), amino acids and derivatives (95), lipids (77) and phenylpropanoids (71) accounted for a larger number, as shown in Fig. 2-D. At the same time, in order to compare the differences in the contents of metabolites between different samples, we calibrated the mass spectral peaks of each metabolite detected in different samples to obtain the relative contents of metabolites in each sample (Supplementary Table S1).

To verify the repeatability and reliability of the detection method, we performed overlapping display analysis on the total ion current (TIC) graphs of the quality control samples under different polarities. The results showed that the TIC curves for metabolite detection have high overlap, that is, the retention time and peak intensity are consistent, indicating that the signal stability is better when the mass spectrometer detects the same sample at different times (Fig. 2-A and B). Furthermore, to detect consistency between biological replications, correlation analysis was performed on all samples. The results showed that with the exception of HN-3, the correlation coefficients of all biological replications were greater than 0.9, which indicated that the replications were good (Fig. 2-C).

3.3 Metabolic profile analysis and biomarker screening of CIF from different habitats

3.3.1 PCA and clustering analysis of CIF

In this experiment, PCA was performed on all samples to obtain a preliminary understanding of the degree of variation between the samples. The results showed that principal component 1 and principal component 2 can explain 21.80% and 18.45% of the differences in metabolites of all samples, respectively. Through the PCA score map (Fig. 3A), it can be further found that there were obvious differences in CIF from different origins. These differences in metabolic levels had obvious correlation with the geographical distribution of mountains. For example, MC, HN, and PY samples belonging to the Ta-pieh Mountains are relatively close, and the SN and CG samples from the Qinling Mountains are clustered together, while the PL sample in the Taihang Mountain area was separated. By drawing a heat map and performing cluster analysis (Fig. 3B), it was found that the classification results were similar to PCA. It is worth noting that the NJ sample was clustered separately, which was the most different from other samples.

3.3.2 OPLS-DA and biomarkers screening of CIF

In addition to the PCA in the unsupervised mode, we also performed OPLS-DA in the supervised mode to further screen for significantly changed metabolites. Through the OPLS-DA score map (Fig. 3C), a sample classification pattern consistent with the PCA results was found, which once again verified that the differences in the metabolic components of CIF are clearly related to the geographical distribution. The interpretation capacity parameters $R^2Y = 0.873$ and the prediction capacity parameters $Q^2 = 0.794$ of the OPLS-DA model indicate that the model had high interpretation and prediction rates, and the classification effect was significantly better than PCA. In order to intuitively show the contribution rate of each metabolite to the grouping, S-plot was made according to the results of OPLS-DA (Fig. 3D). The top five metabolites that contributed the most to the classification were obtained from the results of the S-plot. They were angelicin, N-cinnamoyl serotonin, propyl gallate, 6-aminopenicillanic acid and melatonin. The specific data are shown in Table 2.

Table 2
The biomarker approach for distinguishing CIF from different habitats

Index	Compounds	Class	p[1]	p(corr)[1]	VIP
pmf0558	Angelicin	Phenylpropanoids	77.07	0.79	2.05
pmb0772	N-Cinnamoyl serotonin	Alkaloids	66.13	0.66	1.69
pmf0413	Propyl gallate	Organic acids	64.02	0.75	1.93
pmf0571	6-Aminopenicillanic acid	Others	-76.86	-0.82	2.11
pme1450	Melatonin	Alkaloids	-64.37	-0.84	2.17

3.3.3 Difference analysis of classified metabolites of CIF from different habitats

In the previous part, we conducted cluster heat map analysis for individual metabolites. In order to further explore the accumulation differences of different categories of metabolites, we calculated the total ionic intensity of each category of metabolites and generated a heat map (Fig. 3E). It was found that the NJ sample (Juhuanao) was still clustered separately compared to other samples and the contents of quinones, flavonoids, sterides, lipids and carbohydrates were relatively abundant, while the phenylpropanoid, terpene, indole derivatives and alkaloid contents were relatively low. In contrast to Juhuanao sample, PL sample had abundant alkaloids, terpene, nucleotide and derivatives, amino acid and derivatives, indole derivatives, while the content of sterides, lipids and carbohydrates were low. The common characteristics of CIF from MC, HN and PY were low abundance of flavonoid, quinones, alcohols, amino acid and derivatives, while relatively high abundance of phenolamides and vitamins and derivatives. CG and SN samples were characterized by high phenylpropanoids, alcohols, and carbohydrates.

Since flavonoids are considered to be important active components of CIF, we classified the flavonoids into more detailed categories and determined the relative contents of CIF from different habitats using a column diagram (Fig. 3F). The results showed that the distribution pattern of each classified flavonoids in different samples was not consistent with the total amount of flavonoids. For instance, although the total amount of flavonoids in PL sample was not high, the content of flavone was the highest in the 7 groups of samples. In addition, the contents of flavonols and flavanones in the NJ sample were significantly higher than those in other groups. The highest flavone content was found in the PL sample, followed by NJ and SN. The contents of isoflavones and anthocyanins in CG samples were significantly higher than those in other samples.

3.4 Analysis of significantly changed metabolites in CIF from typical habitats

From the results of PCA and OPLS-DA, CIF from the seven habitats can be divided into four groups at the metabolic level: NJ was one type, PL was one type, SN and CG were one type, and MC, PY and HN were

another type. Then, CG, MC, PL and NJ were selected from the four types of habitats as typical samples for further analysis. A volcano plot was drawn to visually view the differences in the expression levels of metabolites in the two groups of samples and the statistical significance of the differences (Supplementary Fig. S1). We found that the number of SCMs varied from 257 to 323 in different habitats. In general, the number of SCMs in different habitats accounted for approximately 30% – 40% of the total identified metabolites, indicating that the accumulation of metabolites in different habitats was quite different.

The KEGG database was used to annotate the SCMs and perform enrichment analysis (Fig. 4). The results showed that the CIF from four different habitats had an identical similarity in the degree of enrichment of flavonoids components (including flavones, flavanones, flavonols and isoflavonoids) in the biosynthetic pathway. Except for the MC vs PL group, the other five groups showed significant enrichment in the phenylpropanoid biosynthetic pathway. In particular, the SCMs in the MC vs PL and MC vs NJ groups were significantly enriched in the tryptophan metabolic pathway; meanwhile, the NJ vs CG and PL vs NJ groups were significantly enriched in the phenylalanine metabolic pathway.

3.5 Analysis of the metabolic differences between Juhuanao and CIF from other habitats

According to the main use of Juhuanao (usually eaten as a vegetable) and the difference in plant morphology, combined with the results of PCA and cluster analysis in metabolites of all samples, it can be basically confirmed that there was a huge difference between Juhuanao and other CIF. Comparing the NJ sample with those from other habitats, the results showed that PY, CG and PL were the first three samples with the most SCMs compared with the NJ sample, all of which exceeded 300. The Venn diagram was then mapped to explore the repeated SCMs between the different combinations, revealing a total of 62 common SCMs in these six combinations (Fig. 5 and Supplementary Table S2). In the above 62 metabolites, flavones, flavonols and phenylpropanes occupy the majority. Several candidate biomarkers were screened to distinguish Juhuanao from CIF based on the PCA load factor of and the availability of standard reference substances (Table 3).

Table 3
Candidate biomarkers for distinguishing Juhuanao from CIF

Index	Compounds	Class	Accumulation level in Juhuanao
pme0361	Avicularin	Flavonol	High
pme0371	Naringenin 7-O-glucoside (Prunin)	Flavanone	High
pme0374	Isovitexin	Flavone	High
pme3129	Quercetin 4'-O-glucoside (Spiraeoside)	Flavonol	High
pme3211	Quercetin 3-O-glucoside (Isotrifoliin)	Flavonol	High
pmf0179	Narcissoside	Flavonol	High
pma3499	Digitoxigenine	Others	N/A
pme0265	Sebacate	Organic acids	N/A
pme0391	4-Methylumbelliferone	Phenylpropanoids	N/A
pme2993	Scopoletin	Phenylpropanoids	N/A
pme3123	Sinapyl alcohol	Phenylpropanoids	N/A
Note: "High" indicates that the ionic intensity of this metabolite in Juhuanao was at least one order of magnitude higher than that of other samples; "N/A" means that it was not detected in Juhuanao.			

3.6 Correlation analysis of climatic factors and accumulation of metabolites

By searching the China meteorological data service center (<http://data.cma.cn>), we collected ground meteorological data of these seven habitats, mainly including temperature, precipitation, humidity, wind speed and wind direction. Among all climate factors, except annual mean temperature (13.9–16.7°C), annual mean relative humidity (63%-80%), annual extreme maximum temperature (38.3–41.3°C), annual mean temperature daily range (8.5–10.4°C) and annual maximum temperature daily range (20.6–27.3°C), the relative standard deviation (RSD) of other climate factors were all greater than 10%, with great variation. Specific data are shown in Supplementary Table S3. Then, the clustering analysis was conducted on the meteorological data of these seven areas. Through the obtained cluster map and proximity matrix, it can be found that when the distance is 10, the seven habitats can be divided into three categories according to the difference in climate factors: MC, NJ, HN were one category, CG, SN, PY were one category, and PL was one category alone (Fig. 6-A). The similarity coefficients of climate factors from different areas ranged from 0.875 to 0.997, among which SN and CG were the most similar, while PL and HN were the most different. In order to further explore the relationship between climate factors and the accumulation of metabolites, we also conducted cluster analysis on the metabolomic data of CIF and

obtained the proximity matrix. The similarity coefficient of CIF ranged from 0.496 to 0.885, among which HN and MC were the most similar, while NJ and PL had the greatest difference. Then we used mantel test to analyse the correlation between climate factors and metabolites, and the results showed that there was no significant correlation between them ($r = 0.27645$, $p = 0.2079$) (Fig. 6-B). It shows that for *Ch. indicum*, climate factors may not be the main factors affecting the synthesis and accumulation of metabolites.

4. Discussion

4.1 Application of metabolomics in quality evaluation of TCM

In the application process of TCM, the origin of Top-geoherbs of TCM has long been known. The so-called Top-geoherbs of TCM refers to the TCMs produced in a specific region, which have widely recognized excellent quality and are widely used in clinical practice. Of the 500 TCMs commonly used in China, 200 have obvious regional characteristics, and they account for 80% of the total amount of TCM (Huang et al. 2011). The quality formation mechanism of the Top-geoherbs of TCM is mainly affected by genetic factors and environmental modification. The large accumulation of some specific active ingredients in medicinal plants takes place under certain conditions. Therefore, it is an important and meaningful study to select suitable habitats for the accumulation of the main active ingredients in medicinal plants. In this study, metabolomics analysis confirmed that there were significant differences in the metabolite levels of CIF from different regions, such as the abundance of phenylpropanoids, alcohols and carbohydrates in CG and SN samples from the Qinling Mountains, and the abundance of phenolamides, vitamins and derivatives in MC, HN, and PY samples from the Ta-pieh Mountains, which may be the important reason for its geoherbalism.

Due to that the quality of TCMs from different origins is quite different, the origin traceability of TCMs has become a hot research topic at present. With the continuous advancement of instrumental analysis technology, the traceability of TCMs is developing from relying on traditional appearance to biotechnology, such as fingerprinting technology, electronic tongue, electronic nose and DNA barcode (Chen et al. 2019; Hu et al. 2018; Li et al. 2014). Among them, metabolomics has rapidly developed in recent years and has been widely used in the study of the differentiation of TCMs from different origins (Sun et al. 2020; Wang et al. 2020; Yang et al. 2020). Through metabolomics, we not only obtained the metabolic profile characteristics of CIF from different types of producing areas, but also obtained the metabolites that contributed greatly to the classification of CIF from different regions. Importantly, these specific metabolites may be valuable biomarkers for tracing the origin of CIF. In addition, according to the current Chinese Pharmacopoeia standard, it was concluded that the MC and HN samples in the Ta-pieh Mountains had a higher content of index components (linarin), which was consistent with the conclusion that the quality of CIF in the Ta-pieh Mountains was better (Han 2017). However, it is still a question worth discussing whether the quality of CIF can be evaluated only by the content of linarin. For example,

the research results indicated that both flavonoids and caffeoylquinic acids were responsible for the anti-inflammatory effect of CIF through synergetic actions on multi-targets (Tian et al. 2020). Therefore, more comprehensive evaluation indices should be considered in the study of the quality evaluation of TCM. It is worth mentioning that widely targeted metabolomics, due to its characteristics of high throughput and high sensitivity, can make a more comprehensive assessment of the metabolite accumulation level of medicinal plants, thus making up for the disadvantage of a single index component. In conclusion, widely targeted metabolomics has a good application value in evaluating the quality of medicinal plants.

4.2 Main factors affecting the accumulation of metabolites in *Ch. indicum*

Plant secondary metabolites and their content are important indices to evaluate the quality of medicinal materials. The synthesis and accumulation of secondary metabolites are not only determined by their own genetic characteristics but also regulated to a large extent by climate factors such as light, temperature, water and ecological factors such as soil fertility and microorganisms. Both the PCA and OPLS-DA can consistently divide the CIF from the seven habitats into four groups, and this division was strongly correlated with the regional mountains. However, this study found that the correlation between climate factors and metabolites was not significant, which may indicate that the genetic information of *Ch. indicum* has changed greatly in the long-term evolutionary process from a certain level. As reported, *Ch. indicum* is a species with abundant morphological variation, which is not only manifested in the leaf type and flower type but is also abundant in the microscopic morphology, such as pollen, epidermal hairs and stomata (Han 2017). Our research group previously compared the content of metal elements in the soil and CIF from different producing areas, and the results showed that the content of metal elements in the soil from different producing areas was significantly different, the enrichment characteristics of different metal elements were different, among which the enrichment of Cd was higher (Zhang et al. 2018). Another study showed that the contents of total flavonoids and linarin in CIF treated with Cd stress were lower than those in the control group (Zhang et al. 2019). In the previous study, Fang et al. (2010 and 2012) found that there was high genetic variation of *Ch. indicum* at the species level and there was no significant correlation between genetic distance and geographical distance, and also revealed that factors such as insufficient gene exchange, poor seed propagation distance and survival rate, geographical isolation between populations and vegetative reproduction may be the main reasons for the genetic differentiation of *Ch. indicum*. In conclusion, heredity and environment jointly affect the accumulation of metabolites in *Ch. indicum*.

4.3 Discussion on taxonomic status of Juhuanao

Chrysanthemum is a genus with extremely diverse intraspecies variation. Due to its interspecific hybridization, polyploidy, bud transformation and the lack of a clear set of criteria to designate a true species, there have been many controversies on the classification of species in *Chrysanthemum* (Yang et al. 2006; Huang et al. 2016). Wang et al. (1993) found two diploids with different morphologies and karyotypes from *Ch. indicum*, one of which was called NJ diploid due to its limited distribution in Nanjing, China. In some plant taxonomic systems, this NJ diploid is named *Ch. nankingense* (Juhuanao) (Bremer

and Humphries 1993; Dai and Chen 1997). *Ch. indicum* and Juhuanao are both important hybrid materials for ornamental chrysanthemum breeding, and the whole genome of Juhuanao has been sequenced in recent years (Song et al. 2018). At present, it is widely accepted that Juhuanao and *Ch. indicum* are closely related, but whether Juhuanao should be classified as a separate species is still controversial. In the Flora of China, Juhuanao is considered to be the same species as the *Ch. indicum* (Lin and Shi 1983). Chen et al. (1996) believed that because *Ch. indicum* and Juhuanao have different distribution areas and ploidy, their hybrids are highly sterile, so Juhuanao should be regarded as a separate species. Dai and Chen (1997) found through cladological taxonomy that Juhuanao was a branch differentiated from *Ch. indicum* in the early stage, so there was no sufficient basis for it to be a specie alone. Yang et al. (2006) found that Juhuanao is obviously genetically different from other diploid or tetraploid *Ch. indicum*, so this study supported the separation of Juhuanao as a separate species from *Ch. indicum*. In addition, although Juhuanao grew close to the *Ch. indicum* from Nanjing, the molecular marker map showed that there was almost no gene exchange between them, and Juhuanao was the only population with a single cpDNA haplotype (Yang et al. 2006). In this study, the clustering results found that Juhuanao was clustered into a single branch, which showed the biggest difference compared to other *Ch. indicum*, revealing there may be a chemical difference between them. Therefore, at the metabolic level, these results supported the separation of the Juhuanao as a species. Then, whether Juhuanao can be used as CIF in clinical has become a problem to be carefully considered. In this study, several candidate biomarkers were screened to distinguish Juhuanao from CIF, but the specific application of these biomarkers still needs to be further verified. It is possible that multiple biomarkers can be used together to obtain a better discriminant effect. In addition, it was found that the content of alkaloids in Juhuanao was significantly lower than other CIF, which may be one of the reasons for its weak bitterness.

4.4 Implications for cultivation and breeding

Ch. indicum has a long history of application, in addition to China, in Japan, South Korea, India are also widely used (Lim 2014). At present, in addition to medicine, CIF is also often used as tea, spices, food and raw materials of cosmetics, and is listed as a Chinese medicinal material that can be used in health food in China (Song et al. 2020b). Due to the diversity of the application of CIF, the corresponding breeding objectives should be determined for different application directions. For example, the content of active ingredients is the first consideration if it is cultivated as Chinese herbal medicine. If it is developed as a tea product, more attention should be paid to the factors that affect the color and taste of tea soup, such as anthocyanin, volatile oil, etc. If grown as fresh vegetables, emphasis should be placed on reducing bitter substances and extending freshness preservation time. And so on, different uses have different preferences for the phenotype and composition of CIF. In this study, it was found that the metabolite levels of CIF from different producing areas were significantly different, which was determined by the environment and heredity. These metabolic differences provide a variety of possibilities for directional breeding of CIF-related products, and also provide new ideas for further searching for genotypes suitable for the accumulation of certain components and suitable production regions.

5. Conclusion

There were significant differences in the metabolic levels of CIF in different habitats. Metabolomics is a feasible method in the identification of CIF from different habitats. In terms of the degree of enrichment in KEGG, there was a preference for SCM obtained from different habitats with regards to the flavonoid biosynthetic pathway. The five biomarkers (angelicin, N-cinnamoyl serotonin, propyl gallate, 6-aminopenicillanic acid, and melatonin) obtained through metabolomics can effectively solve the problem of the traceability of CIF from different origins and provided a reference for the comprehensive quality assessment of CIF. In addition, it was found that the diploid *Ch. nankingense* (Juhuanao) endemic to Nanjing was the most significantly different from those in other places of production, and candidate biomarkers that could distinguish Juhuanao from common CIF were selected. Both environment and heredity affect the accumulation of metabolites in *Ch. indicum*, and the abundant metabolic differences provide a variety of materials for breeding of *Ch. indicum*.

Declarations

Author contributions Q. Z., Q. G., and T. W. conceived and designed the experiments. Q. Z. and T. W. performed the experiments and written the original draft. Q. Z., J. C., F. Y. and C. Y. analyzed the data and make figures and tables. Q. Z., J. C. and F. Y. contributed to the collection of experimental materials. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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Figures

Figure 1

The distribution of CIF samples from different habitats (A and B) and the differences in the content of linarin (C). Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

green dots denote metabolites with VIP≥1. E: Clustering heat map analysis of classified metabolites in different habitats; F: Ionic intensity of flavonoid metabolites in all samples

Figure 4

KEGG enrichment analysis of SCMs in CIF from typical habitats

Figure 5

Venn diagram analysis of Juhuanao from NJ and samples from other typical habitats

Figure 6

Correlation analysis of climatic factors and accumulation of metabolites in CIF. (A) Dendrogram of hierarchical cluster analysis based on climatic factors in habitats. (B) The relationships between climatic factors and accumulation of metabolites in CIF by mantel tests

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