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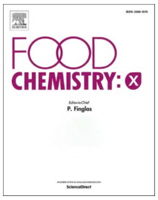

Characterization of a new *Camellia* plant resource with low caffeine and high theobromine for production of a novel natural low-caffeine tea

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ABSTRACT

Yuanbaoshancha (YBSC) is characterized as a new wild tea relative morphologically and phytochemically distinguished from the closest wild tea plants Rongjiangcha (*Camellia yungkiangensis*, RJC) and Tulecha (*Camellia costata*, TLC). YBSC young leaves contain higher tea polyphenol and theobromine contents but lower caffeine and theanine as compared with RJC, TLC, and other tea landraces and modern cultivars. The major alkaloid detected in YBSC, TLC, and RJC is theobromine while caffeine is a minor; the primary catechins in YBSC leaves are non-galloylated catechins, significantly different from *Camellia sinensis* and other low-caffeine tea resources. The unique phytochemical profiles featured YBSC black tea with extremely lower caffeine and higher theobromine, as well as unique flavors and health benefits. This botanical characterization of YBSC and two related low-caffeine wild tea resources lays a foundation for future better utilization for the production of a highly valuable natural low-caffeine/high-theobromine tea.

Chemical compounds: Caffeine (PubChem CID: 2519); Theobromine (PubChem CID: 5429); Catechins (PubChem CID: 9064); Epigallocatechin gallate (PubChem CID: 65064); Theanine (PubChem CID: 439378); Jasmone (PubChem CID: 1549018); cis-3-Hexenyl hexanoate (PubChem CID: 5352543); Hexyl 2-methylbutanoate (PubChem CID: 24838).

1. Introduction

Camellia is a primitive genus belonging to *Theaceae* family. Plants of this genus are mostly produced in Asia and China is believed as the distribution center of *Camellia* trees and hosts >80% of *Camellia* plants. Among them, *C. sinensis* is the most famous and economically important cash crop for producing the most consumed tea drinks; *Camellia japonica* is a world-wide ornamental shrub or flowering tree, and *C. oleifera* is a woody oilseed tree widely cultivated in China and east Asia for the production of high-oleic-acid edible oil (Yang et al., 2022). One of the Sections in *Theaceae* family, Sect. *Thea* (L.) Dyer contains the most *Camellia* plants that are usually used for producing tea and thus have important economic and ecological values. However, there are long outstanding disputes on the botanical classification of *Camellia* plants, due to their diverse and complicated morphological variations, incomplete resource investigation, and inaccurate descriptions of traits and misidentification of *Camellia* plants, classifications on *Camellia* plants are still full of debates, and since Sect. *Thea* was set as an independent section of Genus *Camellia*, the species number of the Sect. *Thea* continues expansion and the classification of Genus Camellia is still under revisions (Chen, 2002; Chen et al., 2000; Chen et al., 2001; Du et al., 1990; Dyer, 1874; Linnaeus, 1753; Ming, 1992; Ming, 2000; Sealy, 1958; Sweet, 1818; Zhang, 1981a; Zhang, 1981b; Zhang, 1984). Therefore, the current taxonomic systems of Sect. *Thea* reflect much confusions, and combination of morphological characteristics, phytochemical classification, and molecular marker-assisted classification became important strategies. Particularly, after the second rounds of national wide surveys on the origin of Sect. *Thea* plants in Yunnan-Guizhou regions in the

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1980s and 1990s, many unidentified plants with *Thea* plant characters were newly discovered and further enriched the diversity of Sect. *Thea*, meanwhile the classification confusions (Wang et al., 2011). Guangxi is located in the southwest of China, in a climatic zone transitioning from tropical to subtropical regions bounded by Yunnan and Guizhou provinces. Because it has a unique geographical and ecological environment suitable for tea plant growth, Guangxi is regarded as one of the important secondary origin centers of tea plant domestication and territory expansion. The tea plant germplasm resources are very rich in Guangxi and some scare wild tea plants were discovered in recent years (Yang, 2021). A wild tea resource that distributed in Yuanbao Mountain with an altitude of over 1000 m above the sea level in Rongshui County, Liuzhou, Guangxi province, is found as a scientifically uncharacterized landrace. This wild tea plant was named as "Yuanbaoshancha" (YBSC). The leaves of YBSC, including buds and young leaves, are obviously different from other local *C. sinensis* plants. The local residents and enterprises have been used YBSC leaves to roast into a black tea with a long-lasting floral fragrance and fresh mellow and sweet tastes. However, the unknown botanical identification and phytochemical property of YBSC have restricted its development.

Among these characteristic secondary metabolites of tea plants making major contributions to tea flavor and health benefits, caffeine (1,3,7-trimethylxanthine) is a key bioactive component present in many popular drinks worldwide, such as tea, coffee, cacao and Yerba mate (Ashihara et al., 2017), due to its strong physiological functions, such as stimulating central neuron system, accelerating metabolic rate, and increasing diuretic effect, and others (Higdon & Frei, 2006). Because of averagely higher level of caffeine in tea shoot tips (~35 mg/g dry tea leaf mass), and the high-dosage intake of caffeine can cause health problems, such as sleeping, learning, increased anxiety and blood pressure, or other health concerns to certain group of people (Smith, 2002; Wikoff et al., 2017), low-caffeine or de-caffeinated tea and coffee are increasingly required and have increasing market shares as an clinging number of consumers pursue caffeine-free or low-caffeine tea/coffee without changing their flavors (Silvarolla et al., 2004). Therefore, breeding of caffeine-free or low-caffeine tea plant varieties is highly desirable and becomes one of the targets in tea plant breeding (Ashihara et al., 2017; Deng et al., 2020). Under the driving force, several low caffeine wild tea relatives have been discovered, such as Cocoa tea (*C. ptilophylla* Chang) (Ishida et al., 2009; Lin et al., 2014), Hongyacha (Jin et al., 2018), and low-caffeine hybrid tea plants (Ogino et al., 2009) and low-caffeine coffea hybrid (Nagai et al., 2008). However, till now these tea resources are not fully utilized for low-caffeine tea production because of various limitations. Therefore, more low-caffeine tea germplasm is highly expected for tea plant breeders and scientific researchers, since it is yet not fully understood how caffeine biosynthesis is exactly evolved in tea lineage.

In order to correctly understand, conserve and make good use of Yuanbaoshancha (YBSC), we first investigated the morphological characteristics. Two wild tea species, Rongjiangcha (RJC) and Tulecha (TLC) naturally distributed in the remote mountainous areas of Guangxi, Guizhou, and Yunnan provinces with similar morphological characters to YBSC plants were also included for comparison. By using morphological, anatomical, and phytochemical analyses of YBSC plants in comparisons with two related wild tea species RJC and TLC, and other local tea landraces and *C. sinensis* cultivars as references, we were able to characterize YBSC plants and posited YBSC as a new variety distinctive from RJC and TLC most likely in a same species of *Camellia* Genus. Based on its phytochemical profile different from other tea plants in tea polyphenols, free amino acids, purine alkaloids, catechins components, particularly in its low caffeine but high theobromine, high non-galloylated catechins but low theanine contents, we further explored the utilization of this unique YBSC plant resource in making highly desirable low-caffeine and high-theobromine tea. Indeed, the YBSC black tea contains very low-caffeine and high-theobromine and meanwhile a robust peach aroma and smooth taste compared with Fudingdabaicha (FDDBC). The study clarifies a novel low-caffeine wild tea resource and improves our understanding of complex Sect. *Thea*, which paves a road towards better development and full utilization of the unique natural tea resources.

2. Materials and methods

2.1. Morphological observation and preliminary identification of YBSC

Yuanbaoshancha (YBSC) was selected as plant material in this study (naturally distributed on the slopes, cols and valleys and streams of Yuanbaoshan at an elevation from 1300 to 1500 m in Antai Township, Rongshui County, Liuzhou City, Guangxi).

According to the morphological description standards (Zhang et al., 2002), the external morphology and internal structure of YBSC in Guangxi were examined in detail. Then it was described and photographed in detail in the order of stem, leaf, inflorescence, floral structure, fruit, seed, flowering period, fruiting period, distribution, growth habitat and so on. And 3 specimens were collected according to the specimen collection and preparation methods. The specimens were stored in the Key Laboratory of Tea Science of Education Ministry of Hunan Agricultural University for reference. Based on the above-mentioned morphological observations, YBSC was identified preliminarily by online search using the Flora Reipublicae Popularis Sinicae (Zhang, 1998).

2.2. Morphological and anatomical analysis

Starting from October/November in 2016 to 2022, YBSC grown in Liuzhou, Guangxi province, RJC in Rongjiang County, Guizhou province, and TLC in Hezhou City, Guangxi province were investigated for sampling, measuring, and recording all detailed morphological and anatomic parameters of leaf, stem, flower and all other >40 selected traits. These included 22 quality traits such as tree type, tree pose, leaf shape, leaf color, leaf tip, leaf base, leaf surface, leaf trichome, flower, calyx trichome hair, petal arrangement, ovary hair, fruit shape, etc. and 19 quantitative traits such as leaf length, leaf width, leaf area, number of leaf vein, anthocaulus length, calyx number, calyx length, calyx width, petal length, petal width, corolla diameter, stigma length, stigma crack depth, filament length, filament number, compartment number of fruit, seed number per fruit compartment, etc., according to the methods described previously (Chen & Yang, 2005; Zhang et al., 2002). 10 individual plants were randomly selected from each resource, and 33 mature leaves in the middle of the stem branches in spring of each year were randomly selected from each plant for observation of the quantitative and qualitative leaf characteristics. About 20 flowers were randomly selected from each resource to examine the quantitative and qualitative characteristics. The mature leaves and flowers of the three resources were selected and brought back to the laboratory as the research materials of the anatomical structure. The epidermis of the leaf was observed by freehand section and the cross section by paraffin section. Ovary cross section, etc. were observed by stereo microscope (MOTIC SMZ-168-TL Stereo Microscope, Microaudie Industrial Group Co., Ltd.).

Preparation and observation methods of paraffin section: Take fresh leaves, cut the leaves into 0.3 cm × 0.3 cm squares with a blade, and immediately put them into the FAA (Formalin-Aceto-Alcohol) fixative (Servicebio, Wuhan, China) to pump and fix them. The fixed material was dehydrated in series with alcohol, transparent in xylene, embedded in paraffin, sectioned in routine, and then stained with Safranin Fast Green Staining Kit (Servicebio, Wuhan, China). Dyeing steps: (1), Paraffin section dewaxing: put the section into xylene I for 20 minxylene II for 20 minabsolute ethanol I for 10 minabsolute ethanol II for 10 min95% alcohol for 5 min90% alcohol for 5 min80% alcohol for 5 min70% alcohol for 5 mindistilled water washing; (2), Safranin stain: stain the section with 1% safranin staining solution for 1–2 h. Wash with tap water to remove excess dye; (3), Decolorization: decolorize the section with 50%, 70%, 80% gradient alcohol for 1 min each; (4), Fast green dyeing: stain the section with 0.5% fast green staining solution for 30–60 s. Decolorization in anhydrous ethanol I for 30 s, and decolorization in anhydrous ethanol II for 1 min; (5), Seal the section: the sections were baked in an oven at 60 °C, and then transparent in xylene for 5 min, and then sealed with neutral gum; (6), Microscope (Nikon Eclipse E100, Nikon, Japan) inspection, image acquisition (Nikon DS-U3, Nikon, Japan) analysis.

Preparation and observation methods of freehand section: Select clean, fresh, and mature leaves, then gently cut a small piece of epidermis with a razor blade, then place the cut epidermis on a glass slide dripped with clean water, covering with a cover glass, and absorb the excess water that overflows from the cover glass. The prepared glass slides with covers were observed under a fluorescence microscope (MOTIC BA400 fluorescence microscope, MOTIC SMZ-168-TL stereo microscope, Microaudie Industrial Group Co., Ltd.) and photographed for further analysis.

The above-mentioned RJC and TLC plants grown in sites were identified and confirmed by Dr. Jiang Daosong (Botanical Research Institute of Hunan Jingran). The details for sampling and descriptions of YBSC, RJC, and TLC tea plants about collection location, elevation, longitude and latitude of the three materials and the serial number of voucher specimen are shown in Supplemental Table S1. Wax leaf specimens of the three resources were all prepared in standard ways. And the voucher specimens were kept in the Key Laboratory of Tea Science of Educational Ministration of Hunan Agricultural University.

2.3. Chemical composition analysis

The tea plant materials in this study, including their sampling locations and time and methods, are shown in Supplemental Table S2. From March to April in 2017, the tender shoots with one bud and two leaves were randomly collected from each tea plant resource without attacked by pests and diseases. Tea materials were fixed by steaming fixing sample method. The specific method was that the collected fresh tender leaves were placed on the boiling steamer, using steam for quick fixing. It was moderate that the leaf color became dark and tender stems were broken without breaking, and the time was 90–120 s. The fixed leaves were spread to room temperature and placed in an oven at 75 °C until they were dry enough. Then the sufficiently dry samples were stored at −20 °C for later use. The moisture determination was conducted according to the national standard GB/T 8304–2013; Water extract from tea leaves was determined referring to the national standard GB/T 8305–2013; Determination of tea polyphenols in tea samples referred to the national standard GB/T 8313–2008; Determination of total free amino acids referred to the national standard GB/T 8314–2013, The contents of flavonoids were determined by using aluminum trichloride colorimetric method (Ma et al., 2008), and the content of soluble sugar was determined by sulfuric acid-anthrone colorimetric assay (Wei et al., 2000).

High performance liquid chromatography (HPLC) was used to determine the contents of catechins, alkaloids, and gallic acid in tea samples. A SHIMADZU HPLC (LC-20AT) equipped with a DAD detector was employed. An ECOSIL C18 Column (4.6 × 150 mm 5 μm C/N EC181546 S/N 4I7501–11) (LUBEX, Japan) was used to separate the tea extracts With a mobile phase A: ultrapure water, and phase B: *N*,*N*-dimethylformamide: methanol: glacial acetic acid = 39.5:2:1.5 (*V*/V/V) in the gradient elution program was as follows: 0–10 min, 9% ~ 14%B; 10–15 min, 14% ~ 23%B; 15–27 min, 23% ~ 36%B; 27–31 min, 36%B; 31–32 min, 36% ~ 9%B; 32–37 min, 9%B. The separation was detected at 278 nm; the column temperature was set at 30 °C, the flow rate as 1 mL/min and the injection volume as 10 μL. Peaks were identified by comparison with the standard compounds (Li, Fu, et al., 2022). The standard compounds such as catechins, alkaloids, and gallic acid were purchased from Sigma Company in the United States.

Measurement of free amino acid contents with HPLC: The tea leaf and standard samples were derivatized with AccQ.Tag™ amino acid derivatization agent from Waters company according to the instructions (Waters, USA). The derivatized amino acid samples were analyzed with a SHIMADZU HPLC (LC-20AT) with a Waters AccQ.Tag™ column Amino Acid Analysis Column (3.9 × 150 mm, 60 Å, 4 μm) (Waters, USA) by using a mobile phase A:10% AccQ.Tag™) solution (AccQ.Tag Chemistry Package, Waters, USA) and phase B: 60% acetonitrile. The gradient elution program was as follows: 0–0.5 min, 0% ~ 2%B; 0.5–15 min, 2% ~ 7%B; 15–19 min, 7% ~ 10%B; 19–32 min, 10% ~ 33%B; 32–34 min, 33% ~ 100%B; 34–37 min, 100%B; 37–39 min, 100% ~ 0% B; 39–42 min, 0%B. The flow was monitored at 248 nm, and the column temperature was set at 37 °C, the flow rate as 1 mL/min, the injection volume as 10 μL. The amino acid standards from British BDH company were used to identify and calibrate the amino acid contents in tea samples.

2.4. Solid-phase microextraction followed by gas chromatograph coupled with mass spectrometry (SPME-GC–MS) analysis of volatile compounds (VOCs) in YBSC and FDDBC black tea

Sample preparation and extraction: Dry black tea materials were weighted and ground into powder. About 1 g of the powder was transferred to a 20 mL head-space vial containing NaCl saturated solution (for inhibiting any enzyme reaction). The vials were sealed with crimp-top caps and TFE-silicone headspace septa (Agilent, Palo Alto, CA, USA). Before SPME analysis, vials were incubated in 60 °C water-bath for 5 min and an 120-μm DVB/CWR/PDMS fiber (Agilent) was inserted and exposed to the headspace of the sample for 15 min at 60 °C.

GC–MS analysis conditions: The desorption of the VOCs from the fiber coating was carried out in the injection port of a GC–MS apparatus, Agilent Model 8890 GC coupled with a 7000D mass spectrometer (Agilent), equipped with a 30 m × 0.25 mm × 0.25 μm DB-5MS (5% phenyl-polymethylsiloxane) capillary column. The injection temperature was set at 250 °C for 5 min in the splitless mode. Helium was used as the carrier gas at a linear velocity of 1.2 mL/min. The oven temperature was programmed from 40 °C (3.5 min), increasing at 10 °C/min to 100 °C, at 7 °C/min to 180 °C, at 25 °C/min to 280 °C, hold for 5 min. Mass spectra was recorded in electron impact (EI) ionisation mode at 70 eV. The quadrupole mass detector, ion source and transfer line temperatures were set, respectively, at 150, 230 and 280 °C. The MS was selected ion monitoring (SIM) mode was used for the identification and quantification of analytes. The identification and quantification of VOCs was carried out by using isotope internal standard (3-Hexanone-2,2,4,4-d4) and comparison with the library with the help from Wuhan Metware Biotechnology Co., LTD (Yuan et al., 2021).

2.5. Data collection and statistical analysis

Principal component analysis (PCA), hierarchical clustering analysis (HCA), and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) were performed using SIMCA-P (V14.1, Umetrics, Umea, Sweden). A variable importance for the projection (VIP) > 1 in the OPLS-DA model served as a differential marker for the sample. The cluster heat map was generated by MeV 4.7.4. The histogram was performed using Excel 2016. The lollipop chart was performed by using a program (https://www.chiplot.online/index.html). Three biological repeats were conducted in GC–MS analysis of VOC profiles. The relative contents of VOCs were calculated with internal standard, and data analyses with heatmap, TopFcBarChart, flavor radarchart, and flavor sankey diagram were carried out by using R package.

All data for quantification experiments were from at least three independent triplicates, the representative photos were shown in the results. For phytochemical profiling, at least three biological samples of triplicates were used for determination. The statistical analysis of the multiple parallel data was carried out by using ANOVA analysis, and the differences between two or three samples were conducted in two-tail comparisons by using Student's *t*-test.

3. Results and discussion

3.1. Morphological description of YBSC plants

The morphological descriptions of YBSC were obtained through observations and measurements of various parameters to accurately define their morphological characters.

YBSC plants are small semi-arbor-type or shrub-type plants with heights of up to 5.7 m. The ground diameters averagely are 20 cm and the height of branch is 3.5 m. The main stem is obvious, and the stem branches spread out at an angle of 20–60°. The color of tender branches is dark reddish brown or purple reddish brown and old branches grayish white. The leaf is thin and leathery, oval, oblong, diamond or obovate ellipse, thinly lanceolate or obovate-lanceolate. Both sides of young leaves are densely covered with white hairs but old leaves are hairless. Flower has 1–2 axillary, the length of anthocaulus is 15–23 mm and hairless; flower diameter is about 25–35 mm with 4–5 sepals, arranged in two rounds, 2 outer and 2 or 3 inner ones and are hairless on both sides. The ovary ovoid is hairless; style 1, 7–10 mm long, 3-lobed linear apex, crack depth is 3–4 mm. The length of fruit stalk is 1.5–2.8 cm, the capsule is flat or spherical or kidney-shaped or triangular, often raised in 2–3 round prismatic, smooth, glossy, 1.2–2.5 cm in diameter, 1.5–1.8 cm in height, woody peel, dust-color when dry, 1.2–1.5 mm thick, 2–3 pieces split from the upper part; seed 1–6, round or nearly round or with 1–2 planes, diameter 1.0–2.3 cm, thick seed coat, horny, yellowish brown, brown or dark brown, smooth, glossy, rich endosperm. Flowering is from October to November, fruit ripening is from October to December of the following year.

3.2. Identification of YBSC plants

According to the morphological observations described above, the Flora Reipublicae Popularis Sinicae (Zhang, 1998) were used to conduct preliminary online search and identification of YBSC, and Sun Yat-Sen University's Herbarium is searched for similar type specimens of related species. Results show that its morphology is similar to RJC (*C. yungkiangensis*) and TLC (*C. costata*) of Ser. *Gymnogynae* in Sect. *Thea* of Zhang Hongda Classification System. The morphological characteristics of RJC and TLC are retrieved in Supplemental Table S3 (Zhang, 1998). The comprehensive and careful comparisons of these morphological characteristics of YBSC, RJC and TLC plants showed that there were high degrees of similarity in various morphological parameters, mainly in the branch color and wax coat condition, leaf structure, e.g. texture, base, vein, and petiole length, flower structure, bract characteristics, style characteristics, three ovaries, etc. Meanwhile, the morphological differences among these three were also obvious (Supplemental Table S4), including stem branching type (arbor or shrub), leaf size, leaf gloss, bud trichome, leaf serration, sepal size, and sepal hairy, ovary structure and seed number per fruit etc. Geographic distribution of these three types of tea plants showed that YBSC and TLC are only located in Guangxi, but RJC is found in Rongjiang, Guizhou province, in Hekou, Yunnan province, and in Damiaoshan, Guangxi province. All of them are suitable for high-altitude evergreen forests and there is no obvious geographic isolation in reproduction. In 1992, Min Tianlu (Ming, 1992) merged the Sect. *Thea* (4 series, 32 species) in Zhang Hongda's classification system (Zhang, 1981a; Zhang, 1981b; Zhang, 1984) into 12 species, 6 varieties and 4 original varieties. He incorporated RJC into TLC and believed that RJC and TLC in Zhang Hongda's classification system lie in the same position. But later 1990, a different classification proposed by Du (Du et al., 1990) and Chen (Chen et al., 2000) made the identification of YBSC more complicated. The morphological trait differences between YBSC, RJC, and TLC seemed to support their likely being different species in the same Series.

3.3. Morphological and anatomical comparisons of the external morphology of these tea trees

3.3.1. Comparison of the external morphology of the tree and the leaf

The survey results of the external morphological characteristics of the trees and leaves of the three resources are shown in Supplemental Table S5. TLC is a small arbor-type with an obvious main trunk, YBSC plants are mainly shrub-type, containing higher levels of stem branching but some are small arbor-type trees. However, RJC is a typical shrub type with basal stem branching. Both RJC and YBSC plants are in a splayed or half splayed pose, while TLC has an obvious upright tree pose and its trunk is twisted, likely for obtaining more lights and better adaptation to the surrounding environment (Fig. 1A). YBSC and RJC are the middle-leafed types, but most TLC plants are the large-leafed type (Fig. 1B). The leaf length ranges of YBSC and RJC plants are shorter than these of TLC ($P < 0.05$) (Fig. 1B, Fig. 1C, Supplemental Table S5). In terms of leaf shape, RJC is oval or oblong or oblanceolate, YBSC is lanceolate or obovate-lanceolate, but TLC leaves are only lanceolate. In terms of leaf trichome, the apical buds and leaves of TLC are hairless, but the apical buds and young leaves of RJC and YBSC are densely covered with white hairs, although both sides of the old leaves in three tea plants are hairless (Fig. 1D, E, Supplemental Table S5).

3.3.2. Comparison of the external morphology of flower and fruit

Flower and fruit are the reproductive organs of plants. Because they are less affected by the environment in the process of plant growth, propagation and evolution, their traits are more stable than those of vegetative organs, thus used as an important basis for plant system classification. As shown in Supplemental Table S6, despite of many common features in flower and fruit morphology of three tea plants (Fig. 2A, B, C), there are also differences among them (Fig. 2A, B, C). For examples, the length of the anthocaulus of YBSC tea plants is significantly longer than these of RJC (12.2 mm) and TLC (8.6 mm) ($P < 0.05$) (Fig. 2D). The calyx of RJC has sparse hairs, while the calyxes of YBSC and TLC are hairless, and their calyx length and width values are also significantly different ($P < 0.05$) (Fig. 2D). The number and the size of petals of YBSC tea plants is similar to that of RJC, both are significantly larger than that of TLC ($P < 0.05$), so are their petal sizes ($P < 0.05$) (Fig. 2D). The stigma crack depth, the average crack depth of RJC reaches 7.4 mm, which is significantly higher than YBSC tea plants (3.4 mm) and TLC plants (3.5 mm) ($P < 0.05$) (Fig. 2D). The number of stamen and filament length of YBSC tea plants are larger but shorter than these of RJC and TLC ($P < 0.05$) (Fig. 2D). RJC fruits were recorded as 3 compartments with 3 seed in each compartment, different from the description of the model species (Zhang, 1981b). TLC fruits with 1, 2 and 3 compartments during our investigation (Fig. 2C).

3.3.3. Micro-morphology comparison of leaf

The leaf anatomical structures of these three tea plant resources are shown in Fig. 3 and Supplemental Table S7, S8. The morphologies of the leaf lower epidermis cells of the three resources are all similarly irregular, but the undulations of the vertical peripheral wall are different. The vertical wall of lower epidermal cells of RJC has the highest undulation degree, followed by YBSC, and TLC has the smallest undulation degree (Fig. 3A, Supplemental Table S7). In addition, single-celled epidermis hairs are found on the lower epidermis of YBSC tea plant leaves. On the abaxial epidermis of RJC leaves, there are stomatal pits arranged radially by multiple cells, but no epidermis hair is found, neither of them is seen on the abaxial epidermis of TLC (Fig. 3A). The cross-section of their leaves (Fig. 3B) show that there are stone cells in the mesophyll cells of the three resources, but there are drastic differences in the number, distribution, and morphology of stone cells in these tea plants. There are fewer stone cells in the mesophyll cells of YBSC tea plants. it's the stone cells in the mesophyll are like hairy. Most of them are across the palisade tissue and the spongy tissue and connect to the upper and lower epidermis. Most of the stone cells located in the main

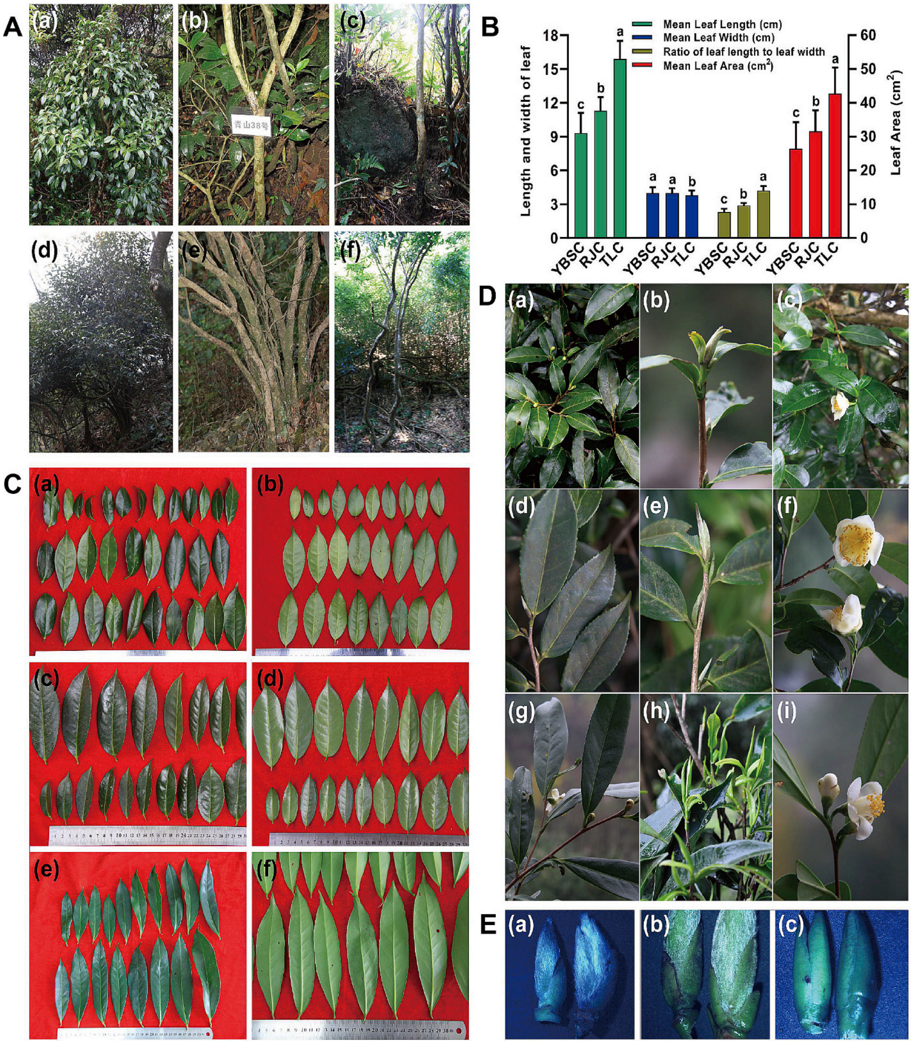


Fig. 1. Morphological and anatomical comparisons of the external morphology of Yuanbaoshancha (YBSC), Rongjiangcha (RJC), and Tulecha (TLC).
A: Plant morphologies of tea plants of YBSC (a, b, c), RJC (d, e), and TLC (f) tea plants.
B: Statistics of leaf traits of tea plants of YBSC, RJC, and TLC.
C: Leaf morphologies of tea plants of YBSC (a, b), RJC (c, d) and TLC(e,f).
D: Sprout, flower and branch morphologies of YBSC (a,b,c), RJC (d,e,f), and TLC (g,h,i) tea plants.
E: Bud trichomes of YBSC (a), RJC (b), and TLC (c) tea plants.
Data are from at least three independent experiments with triplicates. Differences of these parameters among these tea plant resources were analyzed with ANOVA and the different letters show the significant differences.

veins have polymorphic walls, with bone-like branches, and some have star-like branches. There are few stone cells in the mesophyll cells of RJC, which are mainly hairy in morphology, and some have polymorphic walls, with bone-like or star-like branches, whose volume is slightly smaller than that of YBSC tea plants. Most of the stone cells connect the palisade tissue and the spongy tissue, and a few are distributed in the spongy tissue. There are many stone cells with various shapes in the mesophyll cells of TLC. Some are hairy without branches, and some are bone-like branches and star-like branches. But their volumes are smaller than these in YBSC and RJC tea plants. Stone cells of TLC mainly are located in the spongy tissue, and a few connect the spongy tissue and the palisade tissue. The larger number of stone cells present in TLC leaves may be one of the reasons for their harder leaf texture.

The stomata on the abaxial leaf epidermis of three tea resources are also significantly different in the density and the size of guard cell ($P < 0.05$) (Fig. 3C, D). The stomatal density on TLC leaves is the largest, reaching 217/mm^2, YBSC has the second higher stomata density of 185/mm^2, and RJC has the smallest stomata density of 104/mm^2. The sizes of guard cells on YBSC tea leaves are the largest, while these of RJC are the second, and these for TLC leaves are the smallest. From the perspective of the leaf thickness, YBSC tea plants have the thickest leaves, followed by TLC and RJC. The differences among them have reached a significant level ($P < 0.05$). Correspondingly, the one-layer palisade tissue of the three resources of YBSC is the most developed, with a thickness of 97.8 μm, followed by TLC and RJC (Fig. 3B, C, D, Supplemental Table S8). On the contrary, the thickness of spongy tissue of RJC is the most developed and thickest, followed by TLC and YBSC. The upper leaf epidermis stratum corneum (cuticular wax) of YBSC tea plants is the thickest, followed by RJC, and TLC. This is also the reason why the leaf surface of YBSC tea plant is the brightest (Fig. 3C, Supplemental Table S8).

3.4. Phytochemical analyses of YBSC plants in comparison with other sect. Thea plants

3.4.1. The characters of chemical composition of YBSC plants and its comparison with other resources

Extensive phytochemical analyses show that plants in *Camellia*

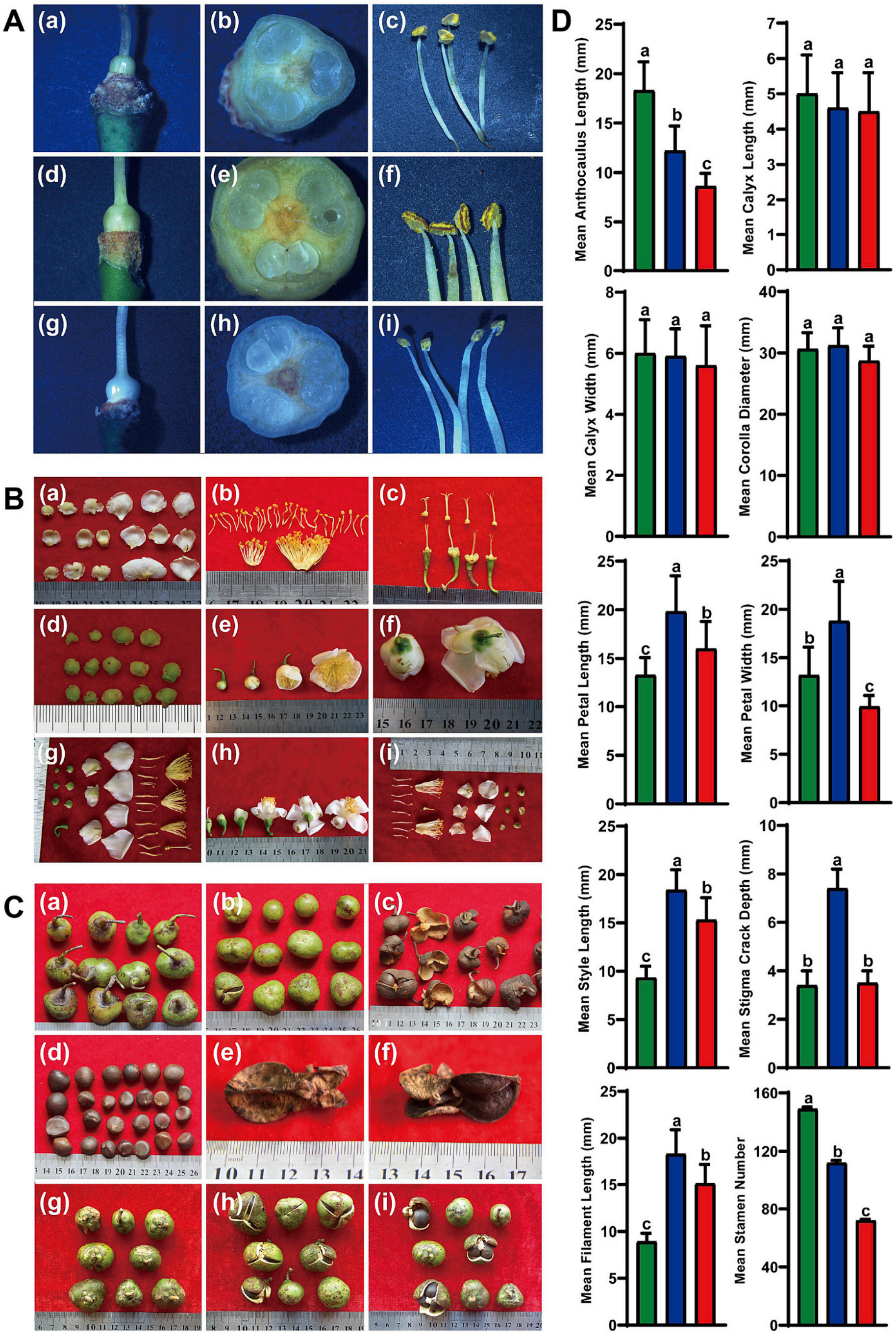


Fig. 2. Comparison of flower and fruit morphologies in three tea plants.

A: The anatomic analysis of ovary cross-section and stamen of YBSC (a-c), RJC(d-f) and TLC (g-i) tea plants. The typical hairless ovary, three-ovary transection and stamen shapes were shown.

B: Flower morphologies of YBSC, RJC and TLC tea plants. (a-d) for YBSC, (e-g) for RJC, and (h-i) for TLC tea plants.

C: Fruit and seed morphologies in YBSC (a-d), RJC (e-f) and TLC (g-i) tea plants.

D: Quantitative comparison of flower parameters of YBSC, RJC and TLC tea plants. Data are from at least three independent experiments with triplicates. Differences of these parameters among these tea plant resources were analyzed with ANOVA and the different letters show the significant differences.

Genus have very strong characteristic phytochemical profiles that are highly consistent with the genetic markers-dependent classification of these plants with highly similar and complicated morphological phenotypes. Also because of the importance of phytochemical profiles to their utilization for tea production, we made a comprehensive examination of their phytochemical profiles in RJC, TLC, and YBSC, in comparison with other local tea landraces, including the population species of Yunnandaye (PS-YNDY), the big tea tree of Yunnan Nannuoshan (BTT-YNNNS) and the big tea tree of Guangxi Jiuwanshan (BTT-GXJWS), which are actually large leaf resources of Yunnan and Guangxi, and local wild tea trees with ages of several hundreds of years. Other local tea cultivars, such as Lingyunbaimao (LYBM), Guire 2, Yaoshanxiulv (YSXL), Yajicha (YJC), Guilv 1, Jiukengzhong (JKZ), and Juhuachun (JHC), were also included for comparison (Supplemental Table S2).

The typical chemical compositions of 13 tea varieties and species are

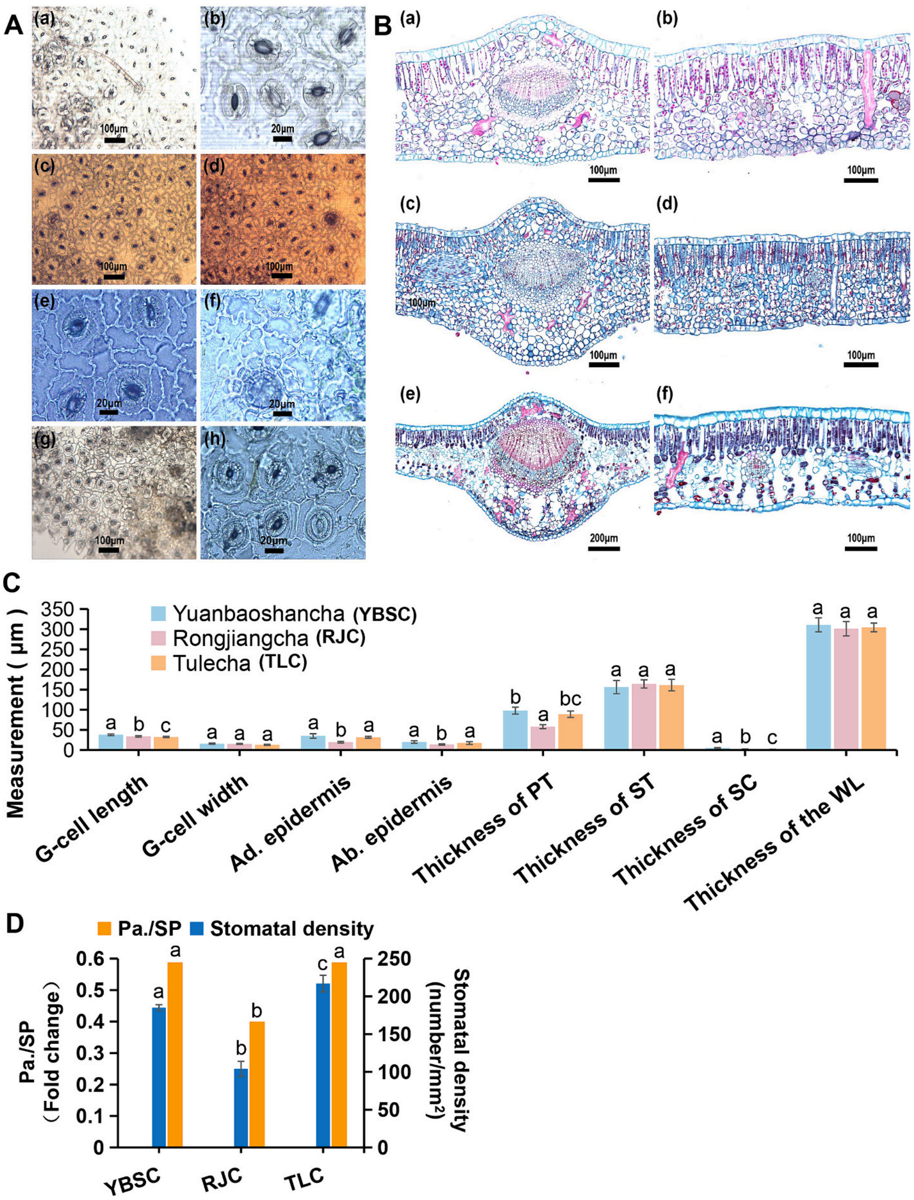


Fig. 3. Comparison of leaf epidermal cells, stomata, surfaces.
A: The stomata morphology on abaxial leaf epidermis from YBSC (a-b), RJC (c-f), and TLC (g-h) tea plants.
B: Comparison on leaf cross-sections of YBSC (a-b), RJC (c-d), and TLC (e-f) tea plant leaves.
C: Quantitative phenotyping of flower structural morphologies. GC (guard cell) length and width; Adaxial (ad) and Abaxial (Ab) epidermis; Thickness of PT (palisade tissues), ST (spongy tissues), SC (stratum corneum) and WL (whole leaf).
D: Quantitative phenotyping of tea leaf thickness and stomata density. Pa/SP: Palisade tissue thickness/spongy tissue thickness ratio. Data are from at least three independent experiments with triplicates. Differences of these parameters among these tea plant resources were analyzed with ANOVA and the different letters show the significant differences.

analyzed with different methods. As shown in Supplemental Table S9 and Table S10, 21 parameters including relative contents leaf water extracts, tea polyphenols, total amino acids, total soluble sugars, alkaloids and catechins have different degrees of variation in 13 resources, of which theophylline has the largest coefficient of variation (177.43%), followed by theobromine (138.08%), DL-C (139.56%), the ratio of non-ester catechins/ester catechins (142.11%), gallic acid (105.31%), and the smallest is water extract (8.19%). It showed that the 13 tested resources have little change range in the content of water extract, but the change range in alkaloids, catechins and other indicators are relatively larger. Catechins and alkaloids are the most important secondary metabolites in Sect. *Thea* plant, and they have important taxonomic value (Du, 1989). It showed that TLC has the lowest catechins content in leaf (~ 6.64%). This is significantly lower than the total catechins of other 12 resources (all >11%), and the highest total catechins in leaves of BTT-YNNNS, reaching 19.87%. The total catechins in RJC and YBSC are 15.63% and 12.55%, respectively (Fig. 4A, Supplemental Table S10). RJC and YBSC leaves contain mainly non-galloylated catechins, 83% and 81%, respectively, which are much higher than these in other 11 tea resources (Fig. 4A, B, Supplemental Table S10). The proportion of non-galloylated catechins in total catechins in other 11 resources is between 20% and 30%, and that for TLC is 9% (Fig. 4B, Supplemental Table S10).

The content of gallic acid in RJC is the highest (0.31%) among 11 tea resources, followed by BTT-YNNNS (0.27%) and PS-YNDY (0.26%), YBSC (0.21%), and TLC (0.16%), respectively. The content of flavonoids of TLC leaves is the lowest (0.27%), RJC (0.61%), YBSC (0.41%) are in

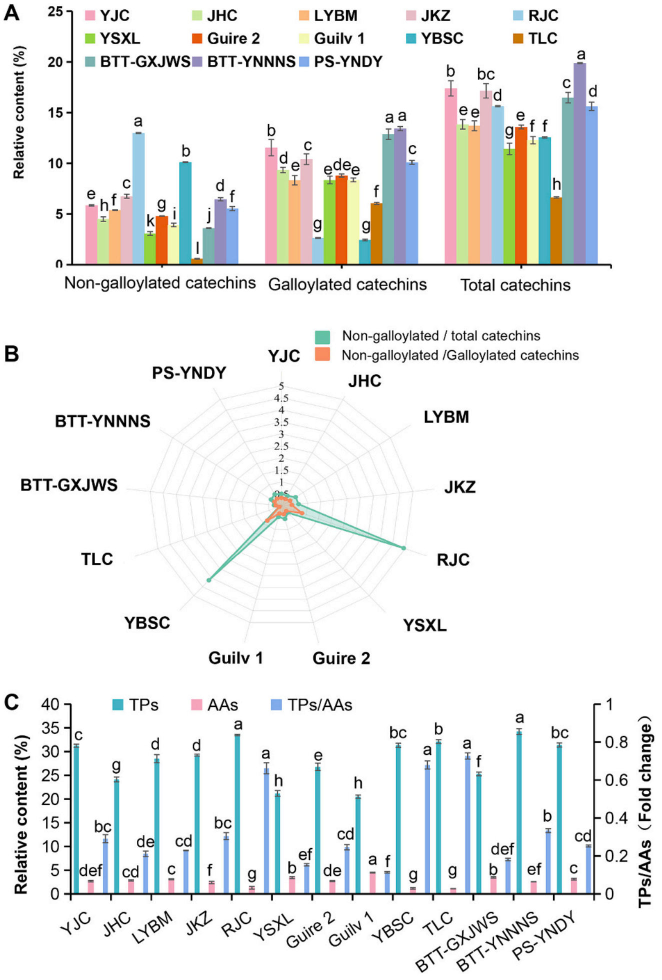


Fig. 4. Analysis of catechins, tea polyphenols, and free amino acids in young leaves of various tea species or varieties.

A: Relative contents of total catechins and percentages of galloylated or non-galloylated catechins in the total catechins determined by HPLC.

B: PCA analyses of the ratios of galloylated or non-galloylated catechins in these tea plant resources. Among them, RJC and YBSC have the highest ratios of non-galloylated catechins.

C: Comparisons of total tea polyphenols and free amino acids among these tea resources. YBSC, TLC and RJC also have relative lower total amino acids than other tea varieties and species.

Data are from at least three independent experiments with triplicates, and presented as means ± s.d.. Differences of these phytochemical components among these tea plant resources were analyzed with ANOVA and different letters show the significant differences.

the middle, and JKZ leaves have the highest flavonoids (1.15%), as determined by using the traditional methods. The content of soluble sugars is the highest in YBSC, reaching 10.96%, RJC (6.25%) and TLC (6.45%) at the middle, and the lowest in BTT-GXJWS (3.79%). The content of tea polyphenols is the highest in BTT-YNNNS (34.23%), followed by RJC (33.50%), YBSC (31.33%), and TLC (32.12%). The total free amino acids contents of RJC, TLC, and YBSC are the lowest, 1.30%, 1.11%, and 1.17%, respectively, which are significantly lower than all other 10 resources (all above 2%) ($P < 0.01$) (Supplemental Table S9). The highest free amino acid content is Guilv 1 leaves, reaching 4.50%. Thus, RJC, YBSC, and TLC have higher ratio of tea polyphenols to free amino acids. The ratio of tea polyphenols to free amino acids of these three tea resources are 26.48%, 27.18% and 29.08%, respectively, whereas the ratios of all other 10 resources are all below 15% (Fig. 4C, Supplemental Table S9).

Although the caffeine and theobromine is the main leaf purine alkaloids in young leaves of Sect. *Thea* plants, the differences in their contents among Sect. *Thea* plants are small and comparable if young samples are well-controlled under various conditions (Du, 1989). RJC, TLC, and YBSC have the significantly lower caffeine contents among these 13 tea resources, only 0.10%, 0.03% and 0.39%, respectively, in comparison with the caffeine contents of young leaves in other 10 tea resources, all >2% ($P < 0.01$). However, the contents of theobromine in young leaves of RJC, TLC, and YBSC plants are the highest, reaching 3.77%, 3.70% and 2.84%, respectively, which are much significantly higher than these in young leaves of other 10 resources ($P < 0.01$). The contents of theobromine in young leaves in other tea resources are mostly below 0.30%, except for BTT-YNNNS (0.88%) and PS-YNDY (0.70%). RJC and YBSC young leaves also contain lower levels of theophylline (~ 0.03%), and BTT-YNNNS and PS-YNDY contain ~0.01% theophylline. But theophylline was not detected in all other 9 tea resources (Fig. 5A, B, Supplemental Table S9). HPLC analysis of catechins and alkaloids of RJC, YBSC, and TLC showed that there is an unknown peak at the retention time of 25.5 min (Fig. 5A). Moreover, the peak area of this unknown peak is extremely large in RJC and YBSC, may contribute to the major tea polyphenols of these YBSC and RJC, although their catechins are not high.

The contents of individual free amino acids in young leaves determined with HPLC showed that histidine contents in RJC, TLC and YBSC leaves are significantly higher than these in the other 10 tea resource leaves, whereas contents of proline, theanine, and valine in RJC, YBSC and TLC leaves are significantly lower than these in other 10 tea resources (Fig. 6A, Supplemental Table S11). Especially, theanine contents of RJC, TLC, and YBSC leaves, 2.83, 20.30, and 5.39 mg·100 g^{-1}, respectively, are significantly lower than these in all other 10 tea resources. Among other 10 tea resources, PS-YNDY had the highest theanine content (925.81 mg·100 g^{-1}) and JHC had the lowest theanine content (165.72 mg·100 g^{-1}) (Supplemental Table S11).

Based on these phytochemical analyses, RJC, TLC, and YBSC among these 13 tea resources belong to Ser. *Gymnogynae* (Zhang Hongda system) (Zhang, 1981a; Zhang, 1981b; Zhang, 1984) according to their morphological characters, which are also similarly different in phytochemical composition from other 10 tea resources belonging to the Ser. *Sinenses* (Zhang Hongda system) (Zhang, 1981a; Zhang, 1981b; Zhang, 1984). RJC, TLC, and YBSC have higher theobromine, lower caffeine, lower free amino acids contents, lower theanine contents, and higher ratio of polyphenols to free amino acids than any other 10 tea resources (Supplemental Table S9, Fig. 4C). Furthermore, TLC is very different from RJC and YBSC in total catechins content and compositions of galloylated and non-galloylated catechins (Fig. 4A, B). Heat map analysis results of key tea characteristic metabolites in various tea plant leaves showed that RJC and YBSC are closer in phytochemical profiles (Fig. 6A, 7A).

3.4.2. Principal component analysis (PCA) of chemical composition of different teas

PCA on phytochemical compositions, including catechins and free amino acid composition in 13 tea resources clearly showed that YBSC, RJC, and TLC share common characters and are often clustered together, providing a phytochemical classification support for their botanic classification into a same Series, which is consistent with their similar morphological characters (Fig. 6B, 7B). The analysis divided RJC, YBSC and TLC into one group, and other tea resources into another group, and differences in non-volatile quality components in between the two groups were analyzed by using OPLS-DA (Fig. 7C). OPLS-DA analysis screened some different compounds (VIP > 1) between the two groups (Fig. 7D). The HCA tree structure showed that these samples had unique characteristics, also supporting that the experiments had good reproducibility (Fig. 7E).

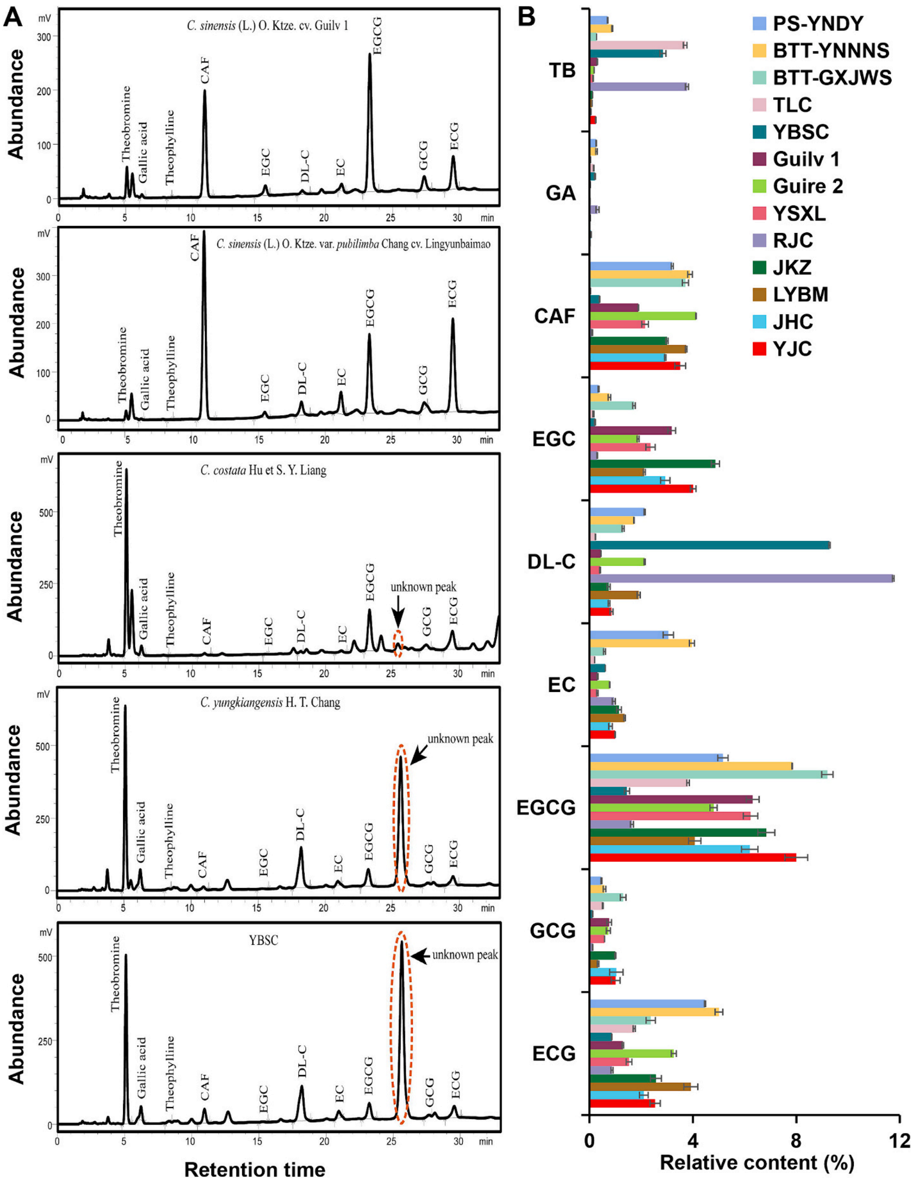


Fig. 5. HPLC determination of catechins and purine alkaloids in young leaves of various tea species or varieties.
A: HPLC chromatograms of catechins and purine alkaloids in tea leaves of *C. sinensis*, *C. sinensis* var. *Pubilimba*, *C. costata*, *C. yungkiangensis*, YBSC. Representative chromatograms were shown for each biological samples.
B: Contents of catechins and alkaloids in young leaves of various tea plants.
Data are from at least three independent experiments with triplicates, and presented as means ± s.d..

3.5. Evolution of YBSC with RJC and TLC

According to the evolution degree of botanical morphology, tea plants can be divided into primitive type, intermediate (transitional) type, and evolutionary type. The systematic evolution of *Camellia* plants appears in the number of carpel or ovary, hair, style, corolla size, fruit development, central axis and other floral and fruit morphological characteristics, as well as tree type, stem branching, leaf size, etc., and these are as the evolution axis (Chen et al., 2006). Zhang Hongda (Zhang, 1981b) divided Sect. *Thea* plants into 4 series according to traits such as the number of ovary compartments, the presence or absence of ovary hairs, and the number of style divisions, including Ser. *Quinquelocularis* with ovary 4–5 compartments, hairless, style 4–5(7) divided and capsule 4–5 compartments; Ser. *Pentastyla* with ovary 4–5 compartments, hairy, style 5 divided, free or apex 5-lobed; Ser. *Gymnogynae* with ovary 3 compartments, hairless, and style 3 divided; Ser. *Sinenses* with ovary 3 compartments, long hair covered, style 3 divided or 3 free. Ser. *Quinquelocularis,* Ser. *Pentastyla,* Ser. *Gymnogynae,* and Ser. *Sinenses* show an increase evolution degree in sequence.

Among TLC, YBSC, and RJC, all have 3-compartment ovary, hairless, and style 3 divided, and they all belong to Ser. *Gymnogynae* (Zhang Hongda system), and are more primitive as compared with *C. sinensis* plants, but they are more evolved than *C. taliensis* and *C. tachangensis* with 5-compartments ovary covered with less hairs (Li, Fu, et al., 2022). The stem branching characters also reflect their evolutionary relationships. TLC is small arbor-type tree with an upright pose; YBSC include both shrub-type and small arbor-type trees with splayed or half splayed poses, but RJC are mostly shrub-type plants with splayed pose (Supplemental Table S5). In terms of leaf size, YBSC and RJC belong to the middle-leafed type, while TLC belongs to the large-leafed type (Supplemental Table S5). These are consistent with the existing studies, that have shown that arbor-type tea plants are primitive, shrub-type tea plants are more evolved; the uniaxial branching is more primitive, whilst the synaxial branching is more evolved. The large-leafed implies

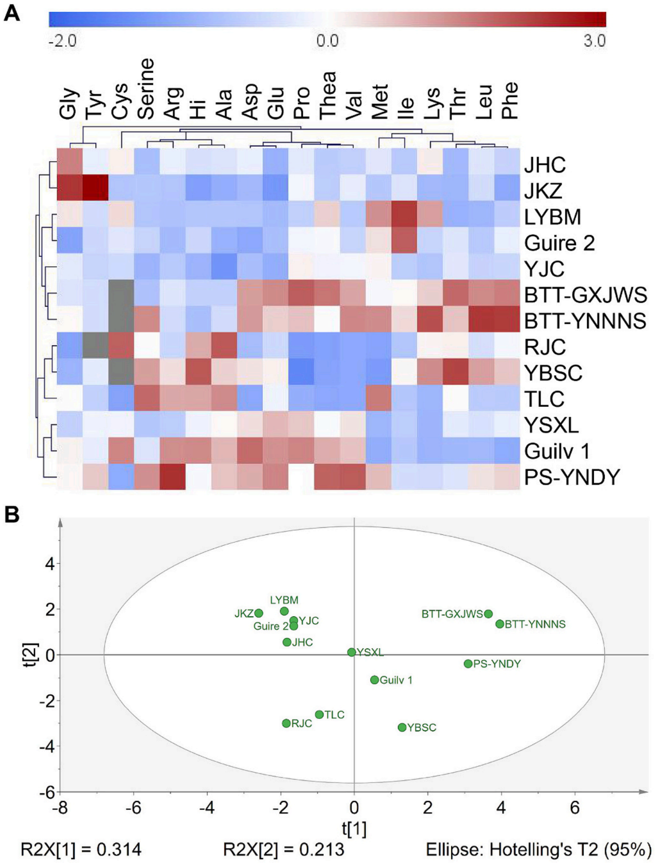


Fig. 6. Comparisons of free amino acids in young leaves of tea varieties and species.
A: Heatmap analysis of various metabolic profiles in tea plant leaves determined by using HPLC.
B: PCA analysis of free amino acids in different tea varieties and species.
Data are from at least three independent experiments with triplicates, and presented as means ± s.d.. The cluster heatmap was generated by using MeV 4.7.4.

the primitive type of tea plants, and the middle-leafed or small-leafed type is the evolved type (Liu, 1981).

It is also believed that thicker leaf stratum corneum is more primitive trait than the thinner leaf stratum corneum, leaf epidermal cells that are wavy and with larger ripples are more primitive than microwave-shaped ones (Chen et al., 2006). Although the leaf stratum corneum of YBSC is thicker, while those of RJC and TLC are relatively thin (Supplemental Table S8). YBSC, RJC, and TLC are more primitive than modern tea cultivars, both Sinensis-type and Assamica-type, in terms of system evolution. Among these three, TLC is more primitive than YBSC and RJC.

3.6. *Discussion on the classification of RJC and TLC*

RJC and TLC as two independent species in Zhang Hongda system, together with 7 other species were unified into *C. gymnogyna* Changin Sect. *Thea* in Flora Reipublicae Popularis Sinicae (Zhang, 1998). We showed that there are huge differences between RJC and TLC in terms of arbor-type or shrub-type of tree, tree pose, leaf morphology, floral morphology and leaf anatomical characteristics, and most of these morphological differences between the two tea plants are up to significant levels ($P < 0.05$). Overall, TLC is posited as more primitive plant than RJC and YBSC resources according to their major morphological traits, because generally, arbor-type stem, globular bud and leaf, and the presence of stone cells are more primitive, whilst shrub-type, trichome-enriched, and the absence of stone cells in tea plants are generally considered as more evolved traits (Li, Fu, et al., 2022).

According to biosynthesis pathway and genome and systematic biology studies, the lack of or lower content of caffeine, but the presence of high level of theobromine in species of *Camellia* genus are regarded as primitive traits as a result of incomplete evolution of the key methyltransferase enzymes. However, higher caffeine and low theobromine may represent evolved traits in tea plant evolution under natural selection and breeder's selection during tea domestication (Li, Ye, et al., 2022). Most modern tea cultivars, including *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica,* contain higher caffeine and much lower theobromine than these wild tea relatives, including *C. tachangensis*, *C. crassicolumna*, *C. taliensis*, *C. gymnogyna,* and *C. pubilimba*, which mostly contain low caffeine and/or high theobromine (Li, Ye, et al., 2022).

Phytochemical analyses indicated that during the evolution and domestication of plants of *Camellia* genus, either Sect. *Thea* or other Sects, the contents of caffeine, galloylated catechins, and total catechins shows an increase trend over the evolution and domestication, but contents of theophylline and theobromine show a decrease trend, and the ratio of tea polyphenols to total amino acids also gradually decreases. Thus, YBSC, RJC, and TLC are evolutionarily more primitive than modern tea cultivars (*C. sinensis*). Moreover, our study further showed that TLC is the most primitive, YBSC is the second, and RJC is the most evolved, as suggested both by their morphological traits and phytochemical characters. Indeed, RJC is distributed in Yunnan, Guangxi, and Guizhou, but others are only in Yunnan and Guangxi.

3.7. *The merits of YBSC black tea with low caffeine and high theobromine*

We further explored the potential utilization of YBSC plant resources. We made benches of black tea with YBSC plant young leaves by using traditional processing technique (Fig. 8A, B, C), with the most widely cultivated *C. sinensis* var. Fudingdabaicha (FDDBC) as a control (Fig. 8D, E, F). The tea samples were assessed in accordance with the methodology of sensory evaluation of tea specified in GB/T 23776–2018. The chemical constituents of quality were also subjected to rigorous testing (Supplemental Table S12, Fig. 8G, H). It was shown that, the YBSC black tea samples have an extremely lower caffeine content (0.20%) and a higher theobromine content (3.98%), in comparison with traditional black tea, represented by FDDBC black tea, which contains higher caffeine content (3.51%) and very low theobromine content (0.13%), similar to all other regular black teas. The sensory evaluation results revealed that the black tea samples exhibited distinct characteristics of a robust peach aroma, a well-balanced and smooth taste, as well as an infusion infused with fruity notes (Fig. 8H).

The profiles of VOCs in different teas usually reflect their flavor characteristics, thus become one of the important parameters for overall sensory quality of tea. We also employed GS-MS to compare the profiles of 1188 VOCs detected in YBSC and FDDBC black tea. For analysis of these VOCs in two tea groups, differential metabolites were determined by absolute $\mathrm{Log_2FC}$ ($|\mathrm{Log_2FC}| \geq 1.0$), with increases by fold change ≥ 2 or decreases by fold change ≤ 0.5 as cutoff for significantly different VOCs between YBSC and FDDBC black tea. >517 differential VOCs were classified into 15 chemical types, including ester, terpenoids, ketone, heterocyclic compound, alcohol, aldehyde, hydrocarbons, acid, amine, phenol, nitrogen compounds, ether, aromatics, halogenated hydrocarbons, and sulfur compounds (Fig. 9A, Supplemental Table S13). The VOCs with the largest differences ranking at top 20 are jasmone, 1-phenylcyclohexylamine, 2-methoxy-4-(methoxymethyl) phenol, fenobucarb, cedrol, hexanedioic acid, isoeugenyl acetate, butylphthalide, biphenyl, *cis*-3-hexenyl pyruvate, 3,5-dichlorophenol, 3,4-dichlorophenol, 3-ethyl-2,5-dimethylpyrazine, 2,6-diethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, 7-methyl-3-octen-2-one, 2-ethylpyrazine, (*S*)-(+)-2-heptanol, 2-heptanol, and tetrahydro-4,6,6-trimethyl-2H-pyran-2-one (Fig. 9B). For analysis how these differential VOCs contribute to

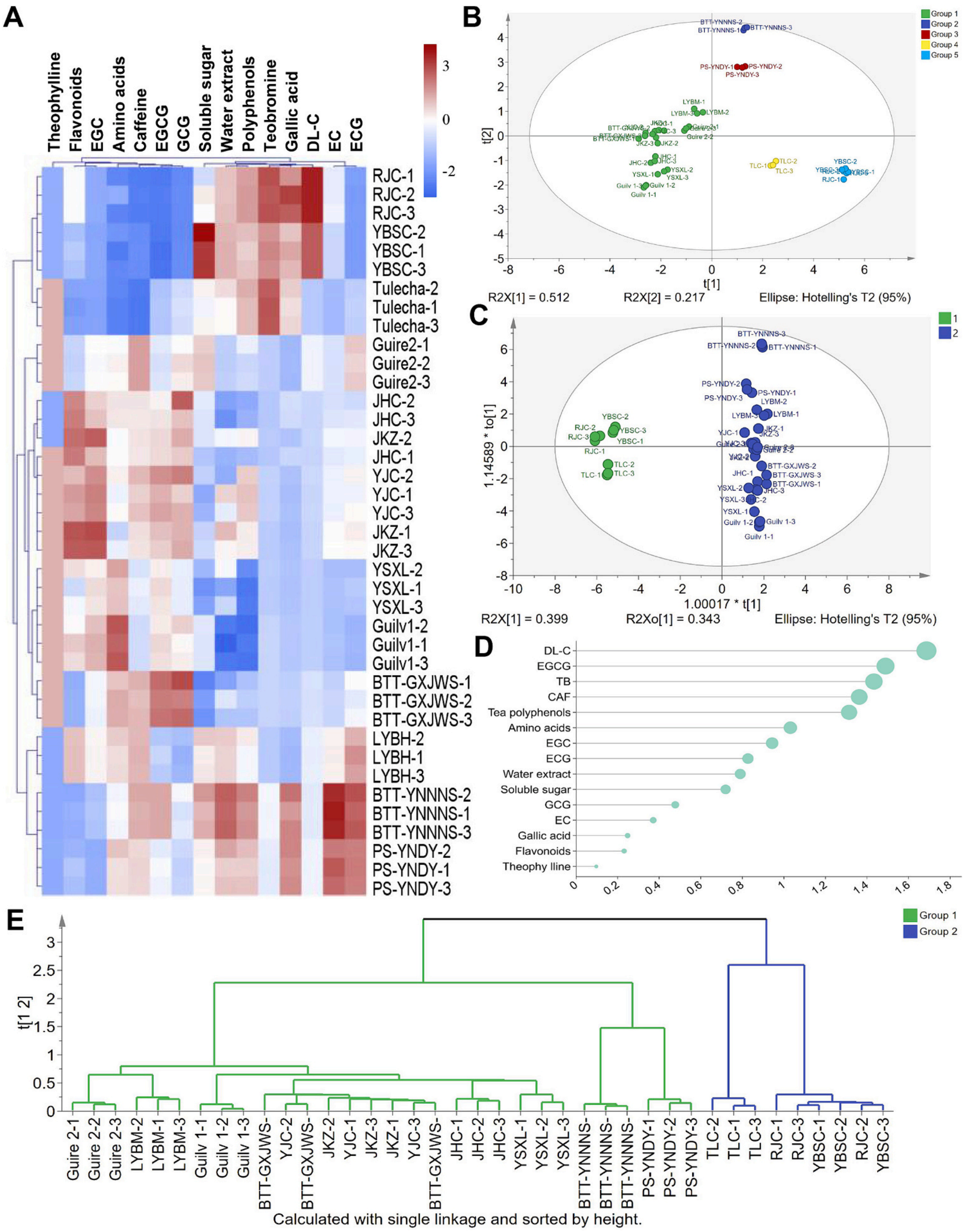


Fig. 7. Comparisons of key tea characteristic metabolites among these tea varieties and species.

A: Heat map analysis of key tea characteristic metabolites in various tea plant leaves. Catechns, purine alkaloids, amino acids, soluble sugar, water extracts, tea polyphenols, and garlic acid contents determined by HPLC and other methods.

B: PCA analysis of the essential parameters for tea materials, such as polyphenols, total amino acids, water extracts, soluble sugars, etc., determined by using traditional metabolic profiling methods.

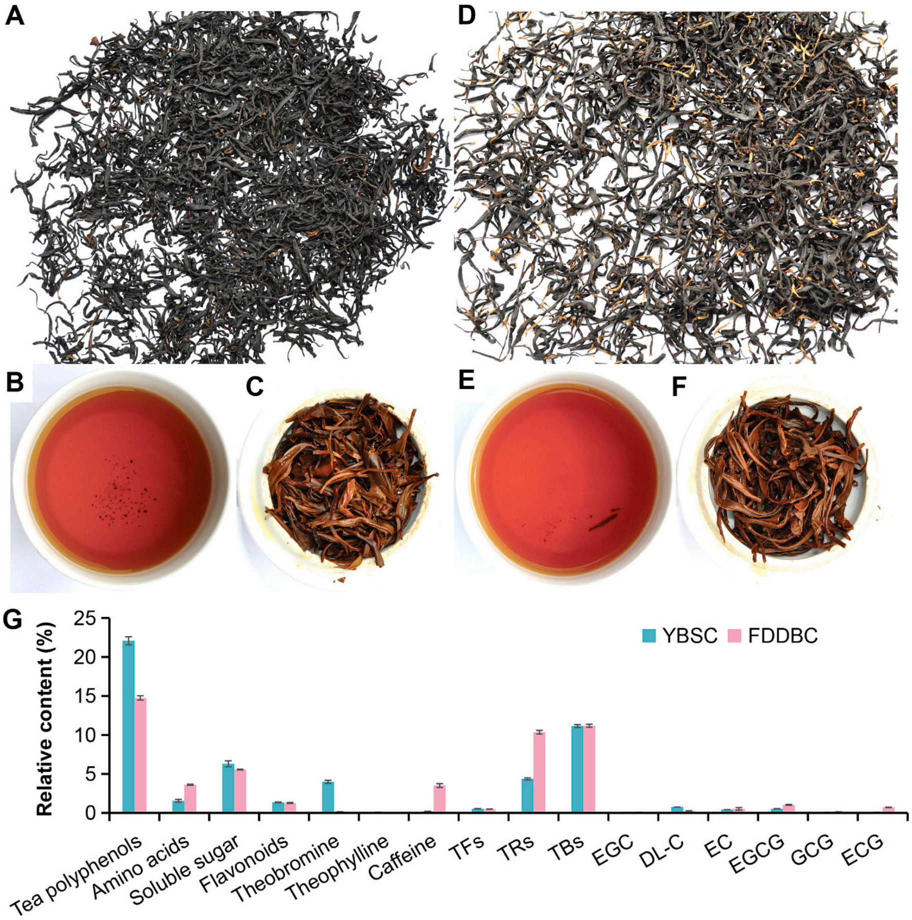
C: OPLS-DA statistical analysisof of catechins and purine alkaloid molecules determined by using HPLC.

D: VIP value of different metabolites in control group and comparison groups. RJC, YBSC and TLC were listed as the control group, and other varieties were listed as comparison group. The area of the circle indicates the size of the VIP value, and the larger the circle, the greater the value.

E: Hierarchical cluster analysis (HCA) of tea characteristic metabolites and critical components and quality parameters of different tea varieties.

Data are from at least three independent experiments with triplicates. The cluster heat map was generated by using MeV 4.7.4. PCA, HCA, and OPLS-DA were performed using SIMCA-P (V14.1).

the different flavors of YBSC and FDDBC black tea, relative odor activity value (rOAV) of these differential VOCs were analyzed. The rOAV is an important parameter to evaluate the contribution degrees of food volatile ingredients to flavor, according to the sensory threshold. The rOAV is the ratio of the relative concentration of each compound to its absolute sensory threshold value in water (Xue et al., 2022). When rOAV ≥1 usually suggests a direct and significant contribution of the compound to food flavor, among 517 differential VOCs evaluated for contributions to characteristic sensory flavor, the first 10 with the largest rOAV ≥1 and sensory contributions to green leaf, sweet, fruity, woody, floral, herbal, nutty, waxy, spicy, rose favors (Fig. 9C, Supplemental Table S14). The VOCs contributing to fruity and floral flavors included 54 and 38 VOCs, both of which had higher contents in YBSC black tea than in FDDBC black tea, mainly include *cis*-3-hexenyl hexanoate, hexyl 2-methylbutanoate, methyl geranate, ethyl anthranilate, beta-cubebene, ethyl 2-furoate, isoeugenyl acetate, 4-phenyl-2-butanol, isobutyl phenylacetate, jasmone, 2-hex-2-enylcyclopentan-1-one, and 1-hexadecanol (Fig. 9D). Thus these VOCs might be identified as characteristic odorants of fruity



H Sensory evaluation of black tea processed from YBSC and FDDBC young leaves

Sample name	Shape	Liquor color	Aroma	Taste	Infused leaves
YBSC	Black bloom, tight shape, even	Orange red, bright	Peach aroma, strong and lasting	Mellow, with the fragrance of peach	Orange red, bright
FDDBC	Tight and heavy, black bloom, slightly tippy	Red and bright	Sweet and lasting	Mellow	Red and slightly bright

Fig. 8. A Black tea processed from the young leaves of Yuanbaoshancha and Fudingdabaicha plant.

A: Appearance of black tea from YBSC; B: Infusion color of YBSC black tea; C: Infused leaves of YBSC black tea; D: Appearance of Fudingdabaicha (FDDBC); E: Infusion color of FDDBC black tea; F: Infused leaves of FDDBC black tea.

G: Comparative measurements of bioactive nutrient ingredients of YBSC and FDDBC black teas. Data are from at least three independent experiments with triplicates.

H: Sensory evaluation of black tea processed from YBSC and FDDBC young leaves.

and floral YBSC black tea. The different VOC profiles in YBSC and FDDBC tea could also more or less reflect the differential accumulation of VOCs in their fresh leaves.

These results indicated that YBSC is an excellent tea resources with great potentials to be processed into high quality, popular low-caffeine black tea. Thus, protection of these natural wild tea germplasm by shoot cutting plantation of YBSC in a large-scale development and utilization plan is undergoing for YBSC tea resource utilization and optimizing of better tea processing to produce low-caffeine popular tea.

Usually tea is made by using Sect. *Thea* plant leaves as they contain rich tea characteristic secondary metabolites such as catechins, caffeine/ theobromine, theanine/ other amino acids for specific tea sensory characters and health benefits (Wei et al., 2018). In regular tea, catechins are the major polyphenols, including epicatechin (EC), D/L-catechin (C), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), and gallocatechin gallate (GCG), among which EGCG is the most abundant catechin molecule, and caffeine is the most important purine alkaloid (Chen & Zhou, 2005; Jin et al., 2018). However, due to high level and the strong bioactivity of caffeine in regular green and black tea, which cause insomnia, higher blood pressure, and anxiety problems (Smith, 2002), increasing demands for low-caffeine, or even caffeine-free or decaffeinated tea and coffee have driven scientists to search for low-caffeine tea and coffea plant germplasms (Nagai et al., 2008; Silvarolla et al., 2004), since artificial decaffeination in tea or coffee manufacturing process may add negative influence on food safety.

Several low-caffeine wild tea relatives have been identified in Fujian, Guangdong, Guizhou, and Guangxi provinces of China, including a wild tea relative Cocoa tea (*C. ptilophylla*) that contains mainly theobromine (up to 6.8%) and almost no caffeine, which was found in Nankunshan, Guangdong and nearby (Ishida et al., 2009; Lin et al., 2014). Its leaves also contain GCG as the major component of catechins (up to 9.88%), instead of EGCG in most modern tea cultivars (Wu et al., 2021; He et al., 2011). Hongyacha was found in southern Fujian, China, also contains

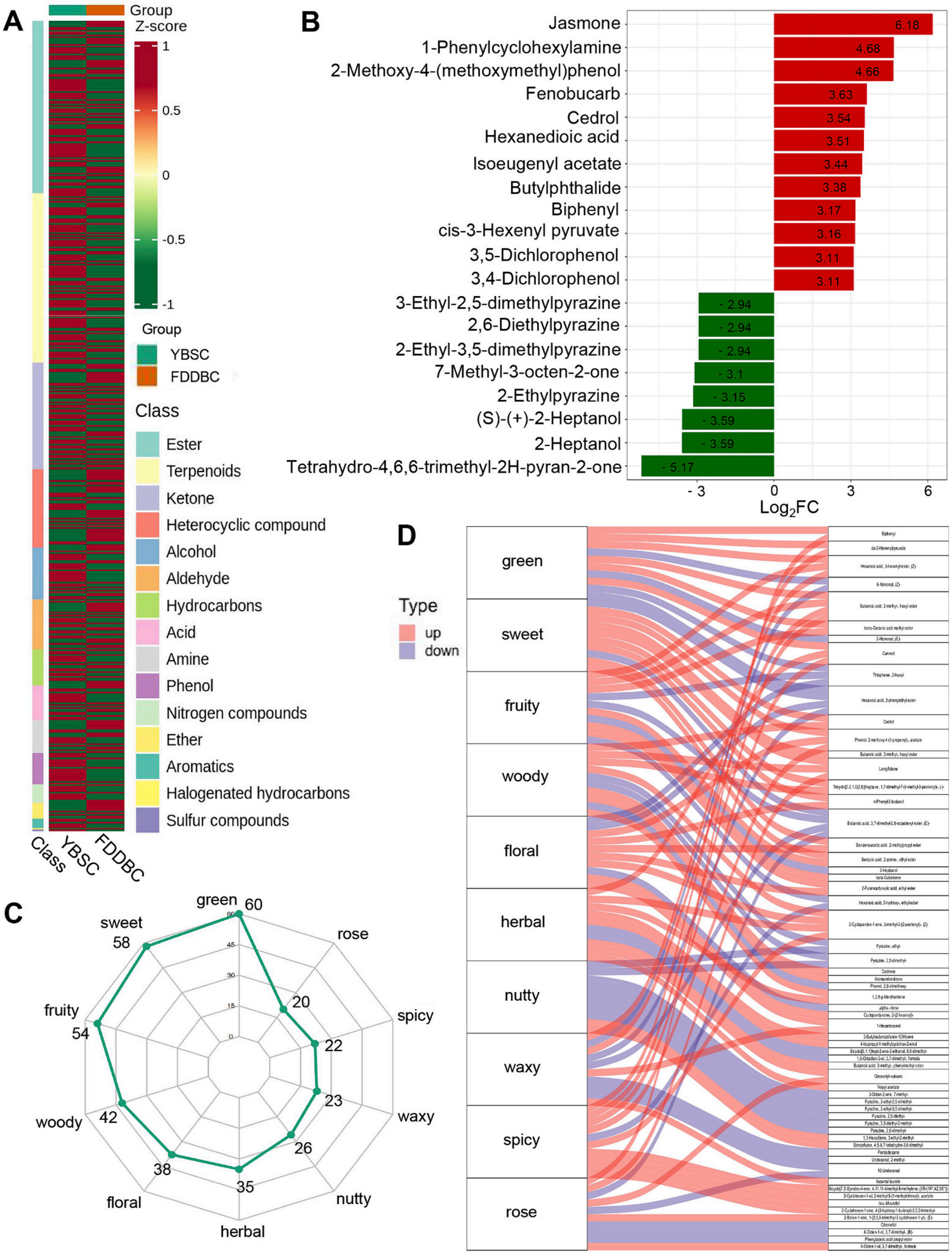


Fig. 9. Analysis of characteristic volatile compounds and different flavors in YBSC and FDDBC black tea processed from their young leaves.

A: Heatmap analysis of VOCs from YBSC and FDDBC black tea profiled with GC–MS. The differential VOCs from YBSC and FDDBC teas were classified into several categories according to chemical structures. Green and red indicated the relative abundance of these VOCs in tea.

B: YBSC vs FDDBC Top FC Bar Chart Index. The X axis represents fold changes of these in YBSC and FDDBC teas with red color refers up-regulation and green represent refers to down-regulation. The Y axis represents the major differential VOCs. The $\log_2$FC values were marked on columns.

C: YBSC vs FDDBC flavor radar chart. The outside circle represents flavor characteristics, green dots and number showed the number of compounds contributing to the flavor.

D: YBSC vs FDDBC flavor sankey Index. The left shows flavor characteristics, The right shows the corresponding VOCs that contribute to the flavor. The lines connecting VOCs to their contributing flavors with red color showing higher contents of VOCs in YBSC than FDDBC, and blue color showing lower contents of the VOCs in YBSC than FDDBC. The tall of the flavor rectangle in the left is corresponding to the contributing VOC number in the right. The taller the left flavor rectangle, more differential VOCs contributing to the flavor. When the number of differential VOCs is >10, then only 10 VOCs with the highest Log_2FC values are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

high theobromine and low caffeine, and GCG as the major catechins, very similar characteristics to Cocoa tea, although its phylogenetic position remains mystery (Jin et al., 2018). *C. ptilophylla* tea has stronger anti-cancer effects than regular tea (Peng et al., 2010; Gao et al., 2020), and also anti-oxidation (Peng et al., 2011; Li et al., 2012), lipid-lowering (Li, Yuan et al., 2016; Li, Liu, et al., 2016) and anti-inflammatory (Lin et al., 2014; Gao et al., 2017). This is probably related to its higher contents of theobromine and GCG (Wu et al., 2021). However, so far the utilization of these low-caffeine tea resources is limited (Wu et al., 2021). Therefore, three novel wild tea resources, RJC, TLC, and YBSC, described here with higher theobromine and low-caffeine, and appropriate levels of catechins could have great potentials to be utilized for producing natural low-caffeine tea with better health benefits. In addition, as novel tea germplasm resource with low caffeine, high theobromine content and unique chemical composition, YBSC plants may provide an excellent genetic resource for the breeding of better low-caffeine tea varieties or materials for more advanced research.

4. Conclusion

In summary, this study reports a first comprehensive botanic characterization of YBSC and two closely related low-caffeine wild tea plants, RJC and TLC. These detailed morphological, anatomic, and phytochemical characterizations and comparisons clearly distinguished YBSC, RJC, and TLC from other tea plants and clarified the confusing disputes. This study thus paves a road towards efficient protection of wild tea resources, appropriate development, and better utilization of these novel tea resources for either making unique low-caffeine tea or producing special valuable materials. By analyzing and comparing the morphological, anatomic characters and phytochemical profiles, it is found that YBSC is evolutionarily closer to TLC and RJC, positioning between the more primitive TLC and more evolved RJC in classification. The rich resources of YBSC also enable the utilization of YBSC young leaves for making a quality low-caffeine black tea. The comprehensive analysis of nutrients and flavors indicated that YBSC could also be used as a high-quality raw material for producing some natural products, such as theobromine, in the deep-processing of tea products, and for more other developmental potentials and utilization values.

CRediT authorship contribution statement

Taolin Chen: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Zhiwen Ge:** Investigation. **Xuemei Yang:** Investigation. **Xifu Wang:** Investigation. **Hao Zuo:** Investigation. **Yinping Liao:** Investigation. **Zhiping Chen:** Investigation. **Zheng Zhang:** Investigation. **Meili Chen:** Writing – review & editing, Supervision, Project administration. **Jian Zhao:** Writing – review & editing, Conceptualization. **Junwu Luo:** Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data involved in this article are all from the research experiments and have not been published in any journal. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Appendix A. Supporting information

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