Assessing the equivalence between traditional medicinal varieties and nontraditional medicinal varieties of Citri reticulata Semen

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Research

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Abstract

Background

Juhe, which is derived from seeds of citrus (*Citri reticulata* Semen), is wildly used as a traditional Chinese medicine. Its original plants have numinous variety, and new varieties are constantly emerging, especially in recent years. The decline in the market share of traditional varieties each year, has resulted in a shortage of Juhe resources, and nontraditional Juhe varieties have been used clinically without research. Thus Juhe resources vary significantly. Furthermore, most of new varieties are hybrids, with significant differences in genes, active ingredients. Therefore, a further research is needed on Nontraditional medicinal varieties.

Methods

In this study, the genetic diversity, shape and size, and flavonoids content of 35 different batches of Juhe samples, from different source, were analyzed base on SRAP molecular markers, a stereo microscope, and HPLC. Then correlation analysis was performed using these data.

Results

The genetic backgrounds of traditional medicinal varieties (TMV) and nontraditional medicinal varieties (N-TMV) differed significantly, and TMV were closely related to some *Citrus* 'Ponkan' samples. Seeds of TMV and N-TMV differ based on shape, size and number. In addition, flavonoid compounds and level in the seeds of TMV and N-TMV also showed a significant difference. Five batches of *C. reticulata* 'Ponkan' samples collected from Zigong, Meishan, Neijiang, Nanchong and Jiangyou were closely related to the traditional medicinal variety *Citrus reticulata* 'Dahongpao',based on genetic relationship, shape and sizes, as well as flavonoids content.

Conclusins:

A total of 5 *C. reticulata* 'Ponkan' samples, including Zigong No. 7, Meishan No. 21, Neijiang No. 27, Nanchong No. 32, and Jiangyou No. 34, were closely related to the traditional medicinal variety *C. reticulata* 'Dahongpao' in genetic relationship, seeds' characteristics and sizes, and flavonoids content. These varieties could serve as a new medicinal variety.

Background

Juhe is the dried ripe seed of *Citrus reticulata* Blanco and its cultivates, which belong to the Rutaceae family[1], and has a long history of medicinal use in China. As early as 1,000 years ago, *Zhujia Bencao* recorded the use of Juhe in the treatment of testicular pain and abdominal pain[2]. Citrus, the source plants of Juhe, are the typical medicinal and edible dual-use varieties, and were cultivated in 2000 BC during Dayu era of China. There are many citrus cultivates, and many new varieties of citrus have emerged due to

intraspecies hybridization and intentional domestication by humans. Thus, it is not surprising that there are significant differences in characteristics, chemical composition and genetic material among these varieties[3]. As one of the auxiliary products of citrus, Juhe's source is closely related to the citrus variety. Thus what are the differences in the medicinal qualities among different varieties of Juhe?

According to the *Chinese Pharmacopoeia* (2015), Juhe has the effects of regulating qi, dissipating binds and relieving pain, and Juhe is often used to treat pain caused by genital diseases, testis swelling and pain, acute mastitis, and mammary hyperplasia[1]. Limonoids and flavonoids are the main active ingredients of Juhe[3, 4], and flavonoids have various physiological functions beneficial to human health, such as anti-oxidation [5], anti-cancer[6], and antibacterial[7]. Juhe is currently widely used in formulas and the Chinese patent medicines, i.e., Huixiang Juhe Pill[8], Juhe Ezhu Granules[9], and Lujiao Juhe soup[10]. Juhe exhibits obvious effects when treating breast hyperplasia[11]. Breast hyperplasia mainly occurs in middle-aged women, accounting for 70% of breast diseases[12], and greatly harming women's physical and mental health. In recent years, Juhe has been used to treat acute mastitis and breast hyperplasia, demonstrating significant effects[13, 14]; thus, significant attention has been given to its medicinal value.

Citrus is the largest fruit in the world, and China is the second largest citrus producer in the world. In total, 19 provinces of China cultivate citrus, such as Jiangxi, Sichuan, and Guangxi[15], and the average annual output in recent years has reached 37.052 million tons[16]. At present, dozens of citrus varieties, such as *C. Tangerina* Hort. Et Tanaka, *C. reticulata* 'Tangerina', *C. reticulata* 'Ponkan', *C. reticulata* 'Dahongpao', *C. reticulata* 'Chachi', *C. Reticulata* Blanco cv. Zhangshuensi, and *C. reticulata* Blanco cv. Kinokuni, are the main source of Juhe. With the continuous appearance of new varieties, significant differences in Juhe have been noted greatly: TMV have been gradually eliminated due to: intolerance to storage, low economic benefits and a lack of market competitiveness. TMV, such as *C. reticulata* 'Dahongpao' and *C. reticulata* 'Tangerina', are gradually replaced by new citrus varieties, such as *C. reticulata* 'Tangerina' and *C. reticulata* 'Ponkan', which have high economic benefits, less and tending to no seeds; only a few fruit farmers plant *C. reticulata* 'Dahongpao'for their own consumption. Juhe quality issues, due to the use of new fruit veriaties, are prominent. Thus as a multisource medicine, traditional Juhe is facing a sharp decline in source and quantity. At the same time, Juhe derived from new varieties is used directly without evaluation, resulting severe quality problems, and difficult to guarantee clinical efficacy.

Citrus is a versatile species. The peel can be used as *C. reticulatae* Pericarpium (Dried Citrus Peel), the dry vascular bundle group can be used as the traditional Chinese medicine Juluo, the pulp can be eaten as fruit, and the seeds are used as Juhe. Most studies on citrus focused on the selection of new fruit varieties, the chemical composition of *C. reticulatae* Pericarpium[17] and its pharmacological effects[18]. Furthermore, research on Juhe has exclusively focused on one type of certain chemical component, such as limonoids[19], but research on whether new varieties can replace TMV is rarely reported. To ensure quality and to identify new medical sources, this study intends to study Juhe based the following aspects: 1) genetic material; 2) shape and size; 3) flavonoids content.

Therefore, this study used different sources of Juhe as research material, employed SRAP molecular marker technology to analyze the genetic diversity combined with stereo microscope technology to quantify the

characteristics and sizes, and applied HPLC technology to measure flavonoids content and quantity. Through the multidimensional evaluation model of genetic diversity-origin-trait-chemical characterization, we explored the distribution and change of active ingredients in different Juhe varieties according to plant kinship, and explained the differences in traits and metabolites of different varieties, and finally be able to:1) establishe a scientific, advanced and practical quality evaluation system to promote and enhance the improvement of quality standards; 2) select excellent Juhe resources and lay the foundation for the sustainable use of Juhe; 3) provide a scientific theoretical basis for the quality evaluation of other medicinal and edible dual-use varieties.

Materials And Methods

Sample collection

From October to December 2015, samples were collected from 14 regions in Sichuan's main citrus producing regions, including Luzhou, Guangyuan, Yibin, Zigong and so on (Fig. 1). Seeds (Fig. 2), picked out from the fruit, and leaves, which were cleaned with absolute ethanol, were stored in a refrigerator at -80 °C until use. The details of sample collection are shown in Table 1.

Analysis of genetic diversity of Juhe

Total DNA was extracted from the leaves according to method 2 in the instruction of the OMEGA Biological Plant DNA Extraction Kit (Omega Bio-tek, Norcross, GA, U.S.), and then its quality was assessed via 2.0% agarose gel electrophoresis. Total DNA was amplified by SRAP-PCR(94 °C for 5 min; 5 cycles of 94 °C for 5 min, 35 °C for 1 min, 72 °C for 1.5 min; 35 cycles of 94 °C for 5 min, 51.2 °C for 1 min, 72 °C for 1.5 min; and 72 °C extension for 10 min) 80 primer pairs (Table 2)[20]. The 20 μL of PCR, mixture contains 10 μL of 2 × Taq PCR MasterMix, 2 μL of primers, 2 μL of DNA template, and 6 μL of dd H_2O. After amplification, electrophoresis was performed on a 2% agarose gel, and products were detected with UV. In total, 22 pairs of primers with good amplification were selected from 80 primers.

Analysis of Juhe shape and size

An X-ray imaging system was used to detect Juhe embryos (Fig. 3), and samples with intact seed embryos were used for subsequent experiments.

Thirty citrus seeds were randomly obtained from each batch of samples. The length of the seed ridge line was recorded as the long, the longest line perpendicular to the seed ridge line was recorded as the wide, and the vertical line from the width to the anti-species was recorded as the height. These three parameters were measured using a stereo microscope. The size of the seed was represented by the sum of the length, width, and height (Fig. 4). The seed's overall variance was used to measure the shape, the smaller the overall variance, the closer the shape is to a sphere (overall variance > 0.05)[21–22]. Otherwise the shape are considered to an ovoid. The following formula was used to calculate the population variance:

$$[3(x_{length}^2 + x_{width}^2 + x_{height}^2) - (x_{length} + x_{width} + x_{height})^2]/3^2$$

Determination of flavonoids

Naringin, hesperidin and neohesperidin levels were assessed. Methanol was used to make standard solutions of 0.14 mg/mL, 0.10 mg/mL, and 0.12 mg/mL. Briefly, 0.5000 g of Juhe powder was mixed with 25 mL methanol. The mixture was shaken well, and then extracted at reflux for 1.5 h in a 70 °C water bath. Then, the solution was cooled and filtered through a 0.45 μm microporous filter to obtain the test solution.

Flavonoids determination was performed on Dikma C18 reversed-phase column (250 mm × 4.6 mm, 5 μm). The solvent system was acetonitrile/0.1% phosphoric acid = at a 20:80 ratio. The following parameters were employed: flow rate, 1.0 mL/min; detection wavelength, 283 nm ; column temperature, 30 °C and sample amount, 10 μL[23].

Statistical analysis

NTsys 2.10e software was used to calculate the Dice genetic similarity coefficient, and the unweighted group paired arithmetic mean method (UPGMA) was used for genetic diversity and cluster analysis. IBM SPSS Statistics 21 software was used for analysis of the following data: SRAP molecularly labeled (1,0) matrix data, seed size and shape, and flavonoids content.

Results

Analysis of Juhe genetic diversity

DNA bands were single, clear, bright, and nontailing, indicating that intact DNA was obtainted at high yields (Fig. 5). In this study, 22 pairs of primers with good amplification efficiency were selected, and genetic polymorphisms of 35 Juhe samples were studied. Obvious differences in SRAP marker polymorphisms were noted. There are 159 clear bands were obtained from this experiment (Fig. 6).

Genetic diversity analysis was performed on the (1,0) matrix data obtained by SRAP labeling of 35 Juhe samples (Fig. 7). The genetic similarity coefficient ranged from 0.73 to 0.94. Sample No. 23 exhibited the lowest relationship with other 34 samples, and the genetic similarity coefficient was 0.73. Base on a genetic similarity coefficient of 0.816, the four samples collected in Nanchong were grouped into one category. The remaining 30 samples had similarity coefficients ranging from 0.82 to 0.93. The genetic relationship is the closest for samples No. 17 and 18, with a genetic similarity coefficient of 0.932. Overall, the relationship between *C.* 'ponkan' and *C.* 'Dahongpao' is the closest, and *Fortunella margarita* (Lour.) Swingle exhibits the lowest relationship with *C.* 'Dahongpao'.

Analysis of Juhe shape and size

The total variance of the 35 batches of orange core samples is greater than 0.05, and the sum of the three dimensions is generally the same (Table 3). The shapes of samples of different varieties from different regions exhibit great differences, and samples of the same variety from different regions also exhibit great differences. The shapes of *C.* 'Ponkan', *Citrus reticulata* and *C. ichangensis* Swingle seeds are similar to those of *C.* 'Dahongpao'.

Seeds from different varieties and origins exhibit no significant differences in the size. The size of *C.* 'Ponkan' seeds is similar to *C.* 'Dahongpao' seeds. However, the seed sizes of *C. sinensis*, *C. reticulata*, *C.* (L.) Osbeck, and *C. ichangensis* Swingle are less similer to that of *C.* 'Dahongpao'.

Determination of flavonoids content

Naringin, hesperidin and neohesperidin levels in 35 Juhe samples were determined (Fig. 8). The content is shown in Table 4, and the chromatogram is shown in Fig. 8. Naringin and neohesperidin levels were relatively low in all samples, and only a limited number of some *C.* 'Dahongpao' samples and *Fortunella margarita* (Lour.) Swingle samples contain naringin. The percentage content of hesperidin exhibits the following trend: *C. sinensis* > *C.* 'Dahongpao'> *C.* 'Ponkan'> *Fortunella margarita* (Lour.) Swingle. Five batches of *C. sinensis* samples (No. 3, No. 22, No. 15, No. 25, and No. 31), 2 batches of *C.* 'Ponkan' (No.34 and No.12) and 1 batch of *C.* 'Dahongpao' (No. 18) contain no neohesperidin.

Correlation analysis of genetic diversity, trait size and flavonoid content in Juhe

SRAP molecular marker clustering analysis showed that the relationship between *C.* 'Dahongpao' from different origins and *C.* 'Ponkan' from different origins was closer than other varieties. The shape and size of different samples were measured using a stereo microscope. Juhe samples of the same variety from different origins exhibits differences in size and shape, and the Juhe samples of different varieties and origins also exhibited differences. HPLC determination of flavonoids showed that only a portion of the *C.* 'Ponkan' samples exhibited composition similar to *C.* 'Dahongpao'.

Cluster analysis was performed on these three sets of data (Fig. 9). The results showed that sample No. 30 had diverged the most from other samples. Among the 12 samples of *C.* 'Dahongpao', except for No. 7, No. 8, and No. 9, had close relationships, and the relationships among samples of No. 14, No. 20, No. 24, and No. 28 were closer. *C.* 'Ponkan' No. 2 and No. 6; No. 12 and No. 13; No. 17, 19, 21, 26, 27, 32 and 34 were grouped into one class separately. The other samples, with the exception of No. 3, No. 10 and No. 14, were grouped into one group, and these samples were relatively dispersed and not closely to each other. In general, the relationship between different varieties and *C.* 'Dahongpao' exhibited the following trend: *C.* 'Ponkan' > *C. sinensis*, *C.* (L.) Osbeck > *C. ichangensis* Swingle, *C. reticulata* and *Fortunella margarita* (Lour.) Swingle.

Discussion

In total, 35 batches of Juhe samples were collected in this study, including 12 TMV, *C.* 'Dahongpao', and 23 N-TMV. The results showed that among 35 samples, *C. sinensis*, *C.* (L.) Osbeck, *C. ichangensis* Swingle, *C. reticulata* and *Fortunella margarita* (Lour.) Swingle significantly differe from the traditional medicinal variety *C.* 'Dahongpao' based on relationship,seed shape and size, and flavonoids content. *C.* 'Ponkan' and *C.* 'Dahongpao' exhibit the highest similarity based on these three aspects, thus, suggesting the need for further researched on this variety as an emerging variety for medical use.

RFLP, RAPD, SSR and SCAR are molecular marker methods usually used to study citrus kinship, but the research found that these research methods have limitations[24], while the SRAP (sequence-related

amplified polymorphism)[25] is Stable, repeatable, short cycle, good polymorphism, which are widely used in the study of genetic diversity and genetic relationship of plant germplasm resources. Therefore, the SRAP molecular marker method was used in this study. Research shows that different varieties of citrus have distant genetic relationships with *C.* 'Dahongpao'. This finding may be attributed to the fact that *Fortunella margarita* (Lour.) Swingle belongs to Fortunella Swingle family, whereas the remaining samples belong to citrus L.. *C. reticulata* is an artificial hybrids that is gererated from Valencia orange as the female parent and Jiangnan orange or zhusha orange as the male parent. *C. sinensis* is a type of *C.* (L.) Osbeck[26], that was originally produced in the United States and were introduced from Morocco and other countries[27] to China in 1965 and later. *C. ichangensis* Swingle is a primitive variety in Sichuan, that potantially originated in the Quaternary Ice Age and is distantly related other citrus varieties[28, 29]. The r close relationship between *C.* 'Ponkan' and *C.* 'Dahongpao' is potentially explained by the fact that both belong to the *C. reticulata* Blanco. Furthermore, *C.* 'Ponkan' was grafted on *C.* 'Dahongpao', and genetic material exchange potentially occurred[30]. The genetic relationship between different variety samples from the same place of origin is closer, thus, the same genetic mutations may occur among different varieties due to similar environmental factors. In addition, citrus varieties easily hybridize[31], thus increasing the similarity in genetic material. Given the long planting history and different genetic mutations, the relationship between *C.* 'Dahongpao' samples is not similar. Natural environmental factors, such as the climate, soil, and altitude of Nanchong, are particularly suitable for the growth of citrus plants. Therefore, samples of different varieties in Nanchong are less related to the other samples, and were divided into a separate branch.

Since ancient times, Chinese medicine scholars believe that the characteristics of Chinese material medica, such as shape, size and odor, were used as identification characteristics and quality standards. However, human-dependent detection is strongly dependent on personal experience and subjectivie. Therefore, stereomicroscopes are often used to observe the morphological structure of some traditional Chinese medicines. For example, Jie Zhang used stereomicroscopes to identify the seeds of *Atractylodes macrocephala* and *Atractylodes lancea*[32]; Xiaolin Li studied the morphological structure of *Salvia miltiorrhiza* seeds using a stereomicroscopes[33]. Each version of the *Chinese Pharmacopoeia* has records regulations on the size and shape of Juhe. Therefore, in this study we use a stereomicroscope to measure the size and shape. This technique presents Juhe's appearance characteristics in a digital form, and avoids subjective error. The study found that Juhe collected from different regions, even in the same variety, exhibit different sizes and shapes, which may be due to different natural factors, such as the sea level, climate conditions, differences in cultivation techniques, cross pollination with other varieties, or different growth periods. Discrepancies in different varieties may be caused by genetic differences.

The main active components of tangerine kernels are limonoids and flavonoids. information on limonoids can be found in our previous reports. Our experiments demonstrated that *C.* 'Ponkan' limonin compounds are closest to *C.* 'Dahongpao'. In this experiment, we first determined hesperidin, naringin, and neohesperidin levels in Juhe. Naringin only exists in some *C.* 'Dahongpao' samples and *Fortunella margarita* (Lour.) Swingle sample. Most *C. sinensis* samples do not contain neohesperidin, This finding may be due to inherent factors such as genetic material and seed maturity[34–35]. Some *C.* 'Dahongpao' samples lack naringin and a portion of *C.* 'Ponkan' samples lack of neohesperidin. These finding may be attributed to their growth environment and studies have shown that some environmental factors, such as light[36],

temperature, irrigation[37], and fertilization[38], can affect flavonoids content in plant seeds. We also found that flavonoids content in *C.* 'Ponkan' and *C.* 'Dahongpao' samples from some places was similar, which was consistent with the research results base on shape and size. This funding may indicate that the seeds' chemical composition was closely related to their physicochemical properties[35], which is consistent with previous research and further illustrates that *C.* 'Ponkan' is the most equivalent to *C.* 'Dahongpao', which proves that *C.* 'Ponkan' is a qualified emerging medical variety.

Cluster analysis revealed close relationships between *C.* 'Dahongpao' samples from Neijiang, Ziyang, Zigong, and Meishan, and the relationships among the *C.* 'Ponkan' samples obtained from these four places are also close. These four cities are geographically adjacent, indicating that mutual introduction may exist. Given similar natural conditions and cultivation techniques, the relationship remains close after many years of cultivation.

In general, most *C.* 'Dahongpao' samples are closely related to the *C.* 'Ponkan' samples, indicating that *C.* 'Ponkan' has the potential to become a new Chinese medicinal material.

Conclusions

In recent years, many new citrus varieties emerged, but new varieties of Juhe were used for medicinal purposes without scientific research. The 2015 edition of *Chinese Pharmacopoeia* does not further specify the cultivation variants that could be used as the source of Juhe. In addition, quality standards could not objectively evaluate the quality differences between different Juhe sources. However, TMV differ greatly from N-TMV based on relationship, traits and active ingredients, TMV have more seeds than N-TMV. Five *C.* 'Ponkan' samples, including Zigong No. 17, Meishan No. 21, Neijiang No. 27, Nanchong No. 32, and JiangyouNo.34 were similar to *C.* 'Dahongpao', can be used as original Juhe varieties for further medicinal research.

To ensure the quality and efficacy of Juhe, this study first explored the equivalence between TMV and N-TMV, and established an evaluation model of "genetic diversity-origin-trait-chemical characterization" from the perspective of modern biology and traditional Chinese medicine. The evaluation model could be used to further improve quality standards of Juhe. The study found that citrus cultivation techniques should be further standardized to reduce quality differences. Moreover, many traditional Chinese medicines are facing a shortage of resources[39, 40], so cultivating new varieties has become a new trend[41, 42], and this study provides idea for the studies of other traditional Chinese medicines.

Additional File

Additional file 1: Table S1. 35 batches of samples' numbers, varieties and collection places. **Table S2.** Calibration curve of three tested compounds. **Table S3.** Stability, repeatability, precision and recovery rate of four tested compounds. **Fig. S1.** The X-ray images of 35 batches of samples.

Abbreviations

TMV: traditional medicinal varieties. N-TMV: nontraditional medicinal varieties. SRAP: sequence-related amplified polymorphism. RFLP: restriction fragment length polymorphism. RAPD: random amplified polymorphic DNA, SSR: simple sequence repeats. SCAR: sequence characterized amplified regions. HPLC: high performance liquid chromatography. *C*. 'Dahongpao': *Citrus reticulat* 'Dahongpao'. *C*. reticulate: *Citrus reticulate. C*. sinensis: *Citrus sinensis. C*. 'Ponkan': *Citrus* 'Ponkan'. *C.* (L.) Osbeck: *Citrus* (L.) Osbeck. *C. ichangensis* Swingle: *Citrus ichangensis* Swingle. *C. Tangerina* Hort. Et Tanaka: *Citrus Tangerina* Hort. Et Tanaka. *C. reticulata* 'Tangerina': *Citrus reticulata* 'Tangerina'. *C. reticulata* 'Chachi': *Citrus reticulata* 'Chachi'. *C. Reticulata* Blanco cv. Zhangshuensi: *Citrus Reticulata* Blanco cv. Zhangshuensi. *C. reticulata* Blanco cv. Kinokuni: *Citrus reticulata* Blanco cv. Kinokuni. ABZ: Aba Zhou. BZ: Bazhong. CD: Chengdu. DY: Deyang. DZ: Dazhou. GA: Guangan. GY: Guangyuan. GZZ: Ganzi Zhou. LS: Leshan. LSZ: Liangshang Zhou. LZ: Luzhou. MY: Mianyang. MS:Meishan. NC: Nanchong. NJ: Neijiang. PZH: Panzhihua. SN:Suining. YA: Yaan. YB:Yibin. ZG: Zigong. ZY: Ziyang

Declarations

Ethics approval and consent to participate

Not applicable

Consent to publish

Not applicable

Availability of data and materials

Most of the data generated of analyzed during the study are included in this article and its Additional file 1.

Competing interests

The authors declare no competing financial interest.

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Author Contributions

Conceptualization, Jin Pei; data curation, Li Wang and Rui Wang; formal analysis, Cuiping Chen; funding acquisition, Qinghua Wu; Investigation, Bin Xian and Li Wang; methodology, Chaoxiang Ren and Jiang Chen; software, Wanting ; Writing – original draft, Bin Xian; writing – review & editing, Qinghua Wu and Jin Pei. All authors have read and approved the manuscript.

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Tables

Table 1 Sample collection

'ariety	place	date	No.	Variety	place	date
. 'Dahongpao'	Qionglai	2015-10-11	19	*C.* 'Ponkan'	Yaan	2015-11-11
. 'Ponkan'	Qionglai	2015-10-11	20	*C.* 'Dahongpao'	Meishan	2015-11-13
. *sinensis*	Qionglai	2015-10-11	21	*C.* 'Ponkan'	Meishan	2015-11-13
. 'Ponkan'	Luzhou	2015-10-14	22	*C. sinensis*	Meishan	2015-11-13
. *reticulata*	Guangyuan	2015-10-22	23	*Fortunella margarita* (Lour.) Swingle	Chengdu	2015-11-19
. 'Ponkan'	Guangyuan	2015-10-22	24	*C.* 'Dahongpao'	Ziyang	2015-11-27
. 'Dahongpao'	Guangyuan	2015-10-22	25	*C. sinensis*	Ziyang	2015-11-27
. 'Dahongpao'	Leshan	2015-10-27	26	*C.* 'Ponkan'	Ziyang	2015-11-27
. 'Ponkan'	Leshan	2015-10-27	27	*C.* 'Ponkan'	Neijiang	2015-11-28
. *sinensis*	Leshan	2015-10-27	28	*C.* 'Dahongpao'	Neijiang	2015-11-28
. 'Dahongpao'	Yibin	2015-10-30	29	*C.* 'Dahongpao'	Nanchong	2015-11-30
. 'Ponkan'1	Yibin	2015-10-30	30	*C. ichangensis*Swingle	Nanchong	2015-11-30
. 'Ponkan'2	Yibin	2015-10-31	31	*C. sinensis*	Nanchong	2015-11-30
. (L.) Osbeck	Yibin	2015-10-31	32	*C.* 'Ponkan'	Nanchong	2015-11-30
. *sinensis*	Yibin	2015-10-31	33	*C.* 'Dahongpao'	Suining	2015-12-01
. 'Dahongpao'	Zigong	2015-11-01	34	*C.* 'Ponkan'	Jiangyou	2015-12-03
. 'Ponkan'	Zigong	2015-11-01	35	*C.* 'Dahongpao'	Jiangyou	2015-12-03
. 'Dahongpao'	Yaan	2015-11-11				

Table 2 The sequence of SRAP primers

Forward-Primer	5’to3’	Reverse-Primer	5’to3’
F1	TGAGTCCAAACCGGATA	R1	GACTGCGTACGAATTAAT
F2	TGAGTCCAAACCGGAGC	R2	GACTGCGTACGAATTTGC
F3	TGAGTCCAAACCGGAAT	R3	GACTGCGTACGAATTGAC
F4	TGAGTCCAAACCGGACC	R4	GACTGCGTACGAATTTGA
F5	TGAGTCCAAACCGGAAG	R5	GACTGCGTACGAATTAAC
F6	TGAGTCCAA ACCGGACA	R6	GACTGCGTACGAATTGCA
F7	TGAGTCCAAACCGGACG	R7	GACTGCGTACGAATTCAA
F8	TGAGTCCAA ACCGGACT	R8	GACTGCGTACGAATTCAC
F9	TGAGTCCAAACCGGAGG		
F10	TGAGTCCAAACCGGAAA		

Table 3 Data of shape, size (n=30)

No.	Long（mm）	Width(mm)	High(mm)	variance	Three-dimensional sum
1	12.33	6.05	4.59	11.96	22.97±0.22
2	11.39	6.39	5.23	7.82	23.02±0.31
3	12.45	6.53	5.61	9.51	24.60±0.35
4	11.39	5.60	4.72	9.37	21.70±0.31
5	13.95	8.37	6.28	13.06	24.16±0.32
6	11.33	6.35	5.17	7.60	22.84±0.33
7	12.92	6.17	5.07	10.96	28.60±0.27
8	12.18	5.31	4.25	13.87	21.75±0.39
9	9.93	5.97	4.78	5.20	20.69±0.31
10	13.09	6.70	5.74	11.33	25.52±0.34
11	11.32	5.80	4.76	8.71	21.89±0.23
12	11.84	6.03	4.50	11.26	22.38±0.41
13	12.46	5.52	4.62	13.2	22.60±0.31
14	11.90	7.02	4.89	9.11	23.81±0.18
15	12.53	6.36	5.13	11.43	24.03±0.37
16	12.35	5.36	4.64	13.45	22.35±0.34
17	10.21	5.68	4.85	5.99	20.75±0.38
18	10.83	5.69	4.65	7.66	21.19±0.24
19	10.77	6.08	4.84	6.97	21.70±0.25
20	12.18	5.78	4.79	11.51	22.76±0.32
21	11.45	5.93	4.82	8.89	22.20±0.30
22	12.13	6.65	5.15	9.54	23.93±0.37
23	10.25	5.49	4.34	6.98	20.08±0.23
24	11.85	5.41	4.24	12.01	21.50±0.29
25	13.47	6.48	4.98	14.66	24.94±0.44
26	10.57	6.16	4.86	6.32	21.59±0.25
27	11.31	5.82	4.84	8.59	21.97±0.20
28	12.33	5.30	4.34	13.14	21.98±0.25
29	11.35	5.56	4.64	10.96	25.44±0.22

30	12.96	7.10	5.37	20.35	26.01±0.39
31	14.72	6.45	4.84	7.83	21.06±0.30
32	10.81	5.57	7.83	9.81	21.55±0.29
33	11.31	5.54	4.50	9.77	21.35±0.24
34	10.84	5.44	4.68	12.73	22.21±0.32
35	12.24	5.49	4.48	8.06	20.97±0.26

Table 4 Flavonoids content of 35 Juhe samples (n=3)

Naringin	Hesperidin	Neohesperidin	No.	Naringin	Hesperidin	Neohesperidin
/	0.0631	0.0103	19	/	0.0340	0.0031
/	0.0143	0.0028	20	0.0093	0.0310	0.0151
/	0.0131	/	21	/	0.0337	0.0031
/	0.0305	0.0052	22	/	0.0189	/
/	0.0299	0.0037	23	0.0392	0.0123	0.0029
/	0.0805	0.0025	24	0.0093	0.0404	0.0167
/	0.0662	0.0095	25	/	0.1792	/
.0139	0.0678	0.0138	26	/	0.0539	0.0023
/	0.0372	0.0039	27	/	0.0543	0.0047
/	0.0329	0.0037	28	/	0.0568	0.0105
/	0.0653	0.0119	29	0.0094	0.0320	0.0179
/	0.0252	/	30	/	0.0331	0.0031
/	0.0305	0.0046	31	/	0.2509	/
/	0.0156	0.0024	32	/	0.0659	0.0028
/	0.0239	/	33	0.0120	0.0527	0.0166
.0068	0.0342	0.0093	34	/	0.0150	/
/	0.0316	0.0039	35	/	0.0328	0.0118
/	0.0403	/				

Figures

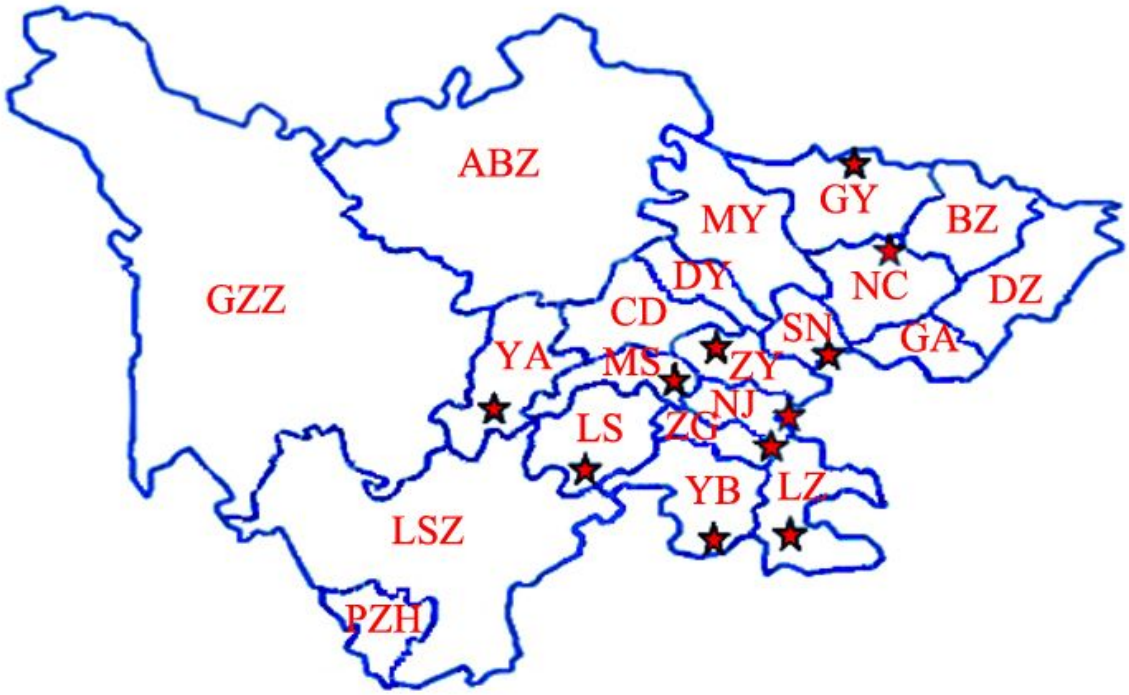

Figure 1

Map of collection place of Juhe samples. Note: ABZ: Aba Zhou; BZ: Bazhong; CD:Chengdu; DY: Deyang; DZ: Dazhou; GA: Guangan; GY: Guangyuan; GZZ: Ganzi Zhou; LS: Leshan; LSZ: Liangshang Zhou; LZ: Luzhou; MY: Mianyang; MS:Meishan; NC: Nanchong; NJ: Neijiang; PZH: Panzhihua; SN:Suining; YA: Yaan; YB:Yibin; ZG: Zigong; ZY: Ziyang. 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.

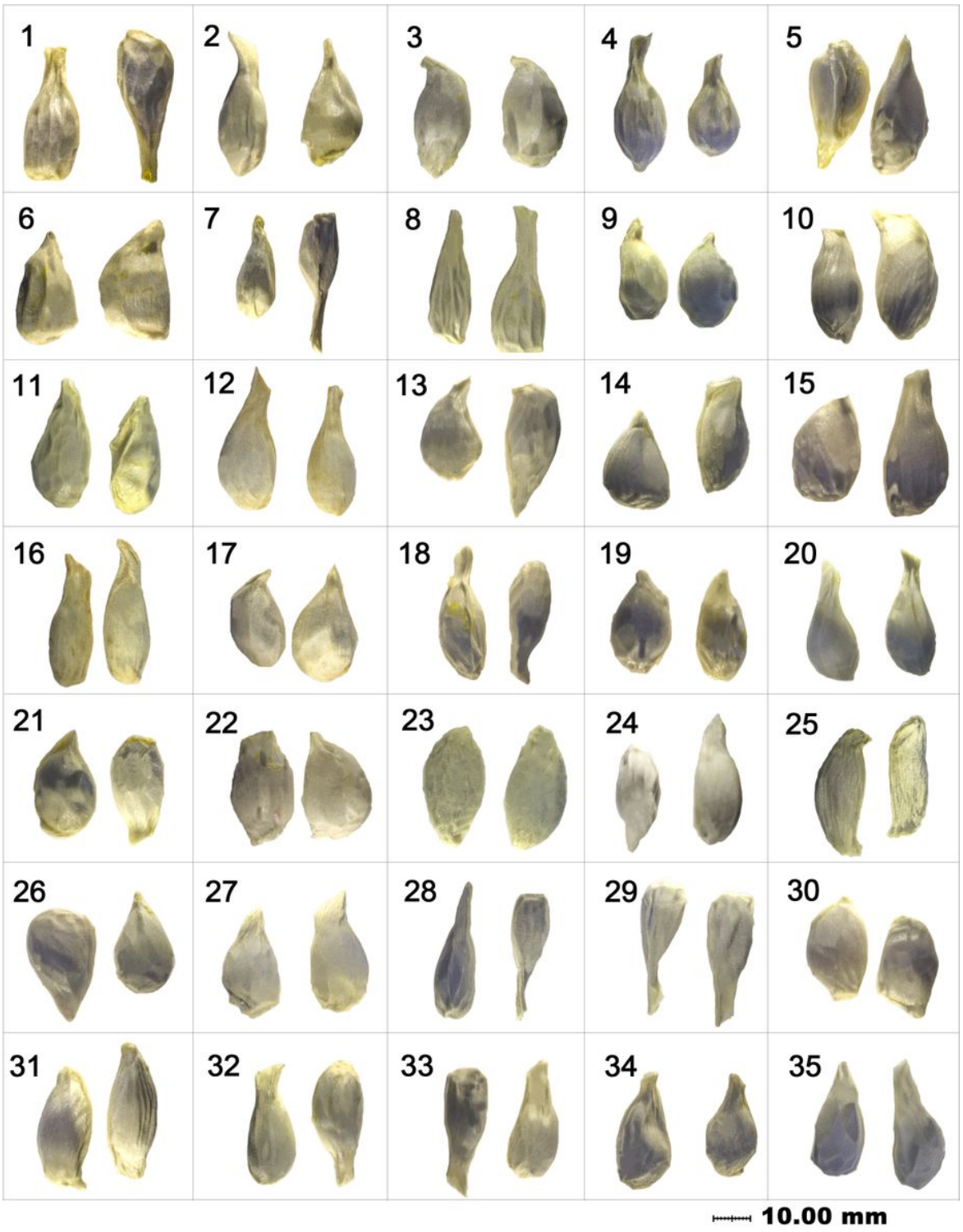

Figure 2

Seeds picture of 35 Juhe samples.

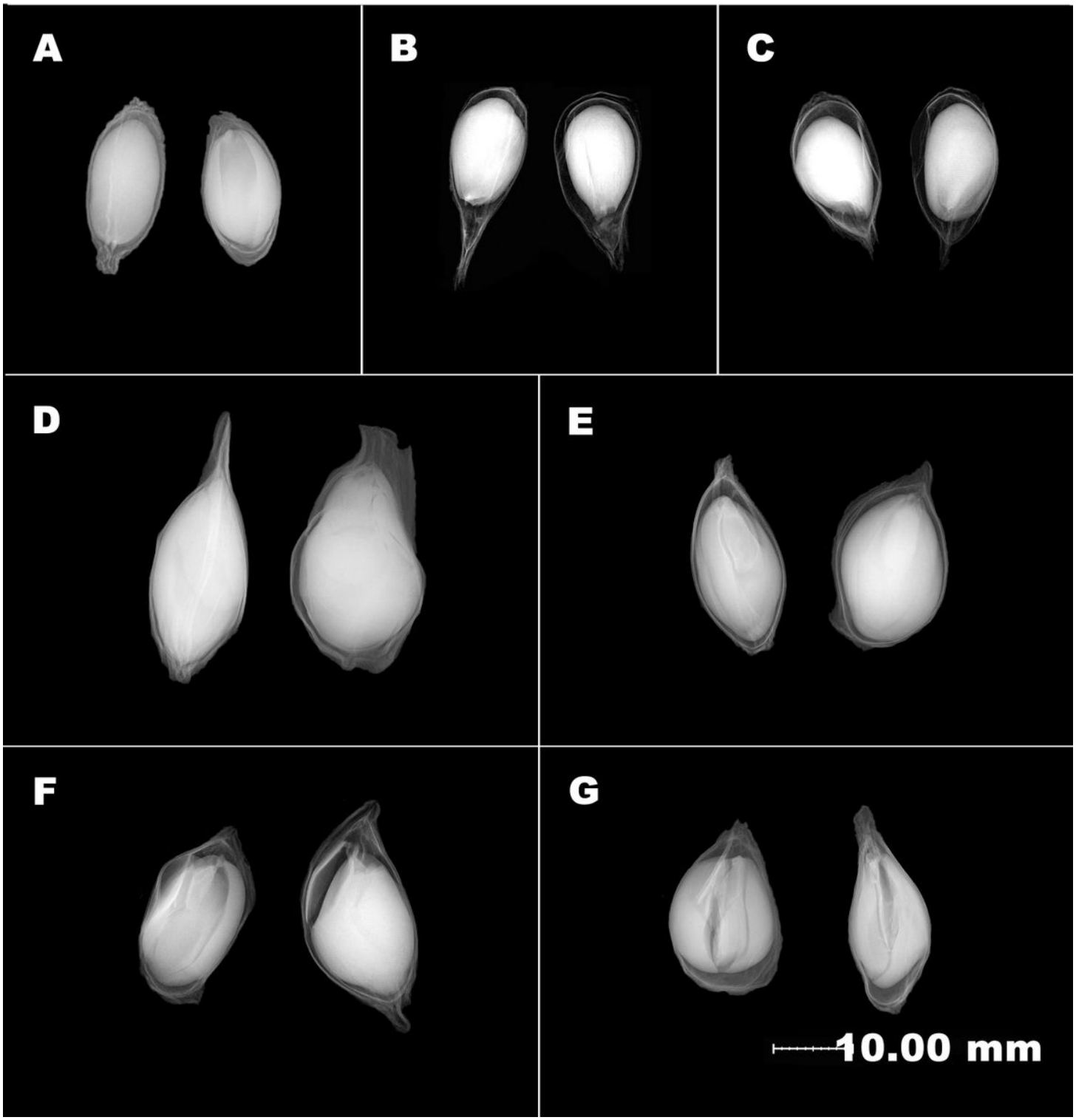

Figure 3

X-ray perspective of Juhe samples. Note: A: seeds of margarita (Lour.) Swingle; B: seeds of C. ‘Dahongpao’; C: seeds of C. ‘Ponkan’; D: seeds of C. reticulate; E: seeds of C. ichangensis Swingle; F: seeds of C. sinensis G: seeds of C. (L.) Osbeck.

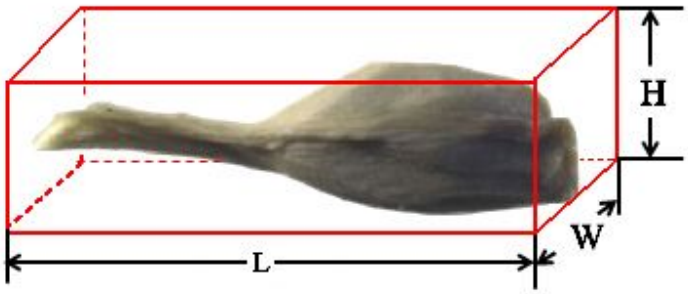

Figure 4

Length, width and height of Juhe samples. Note: L: long; W: width; H: high.

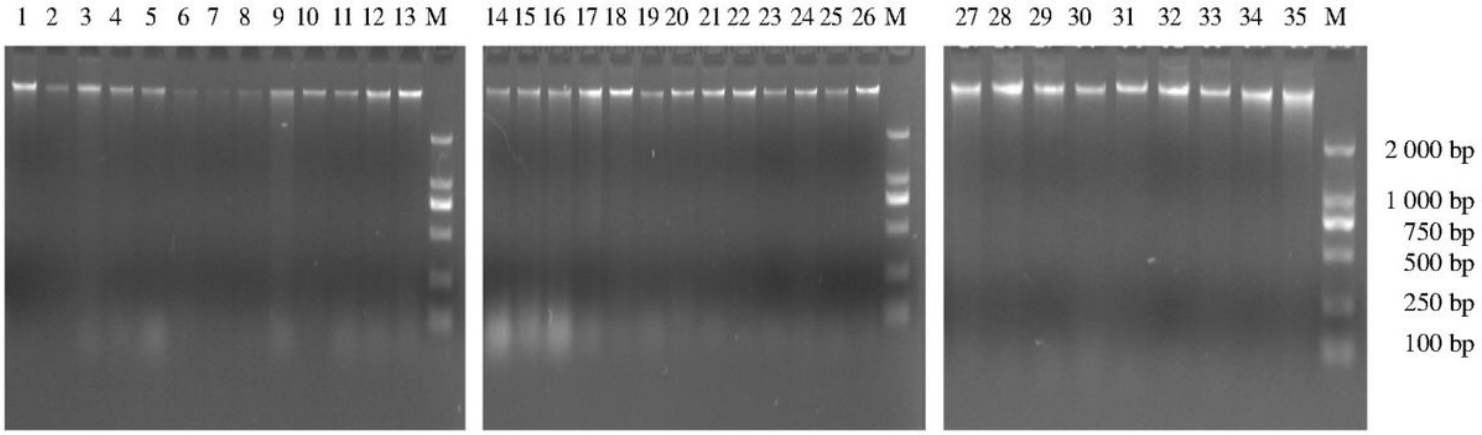

Figure 5

Total DNA electrophoresis

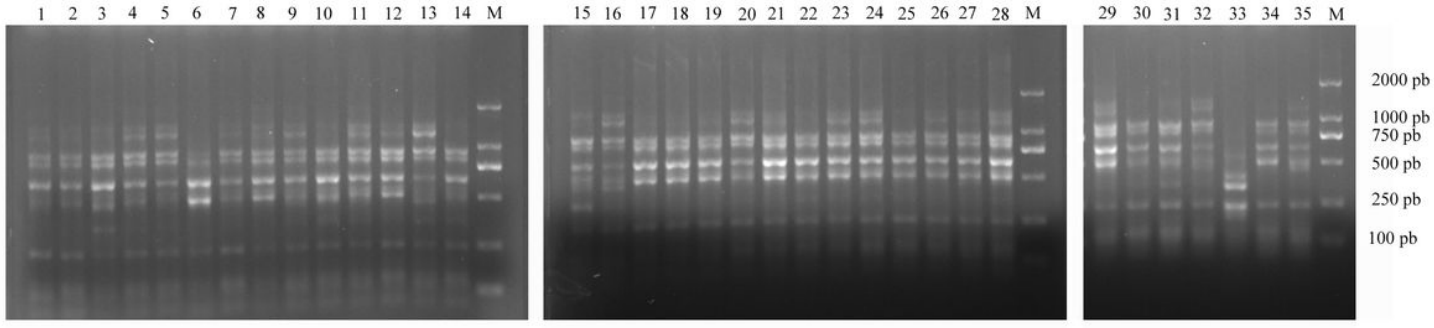

Figure 6

The amplification results electrophoresis of SRAP primer F8-R8

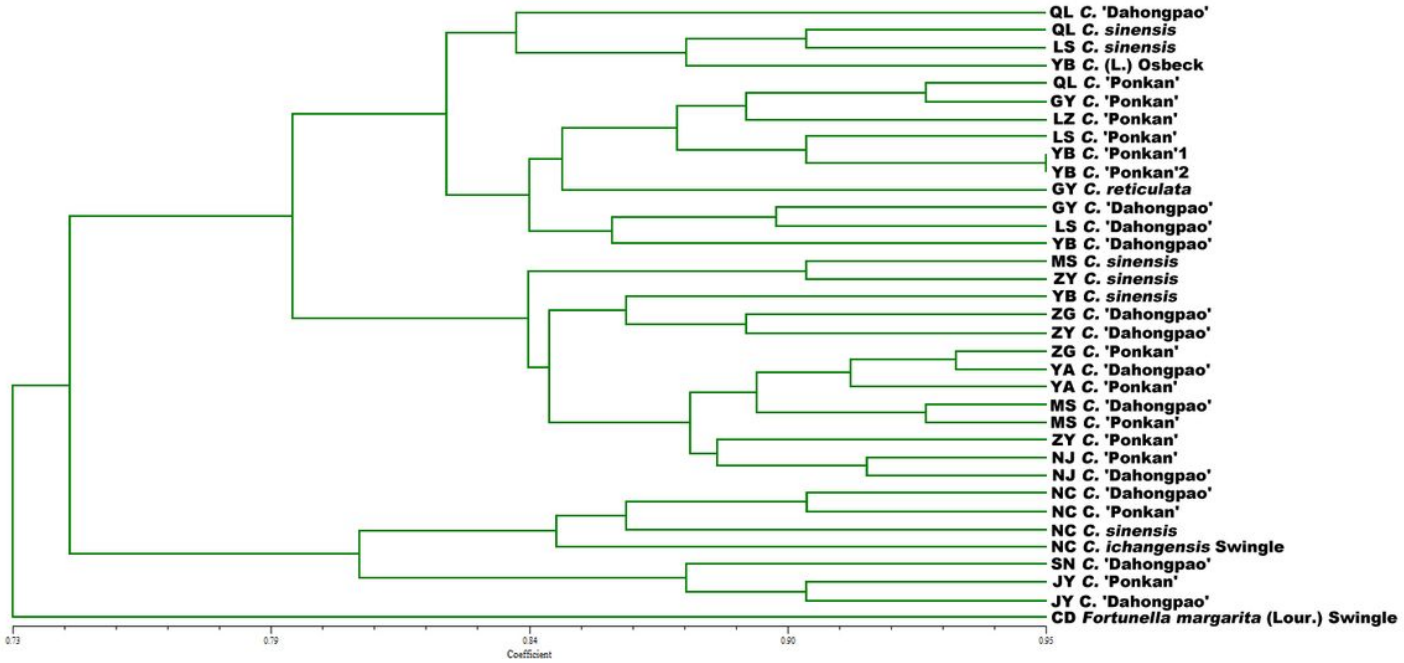

Figure 7

Dendrogram of genetic relationships of 35 Juhe samples using SRAP

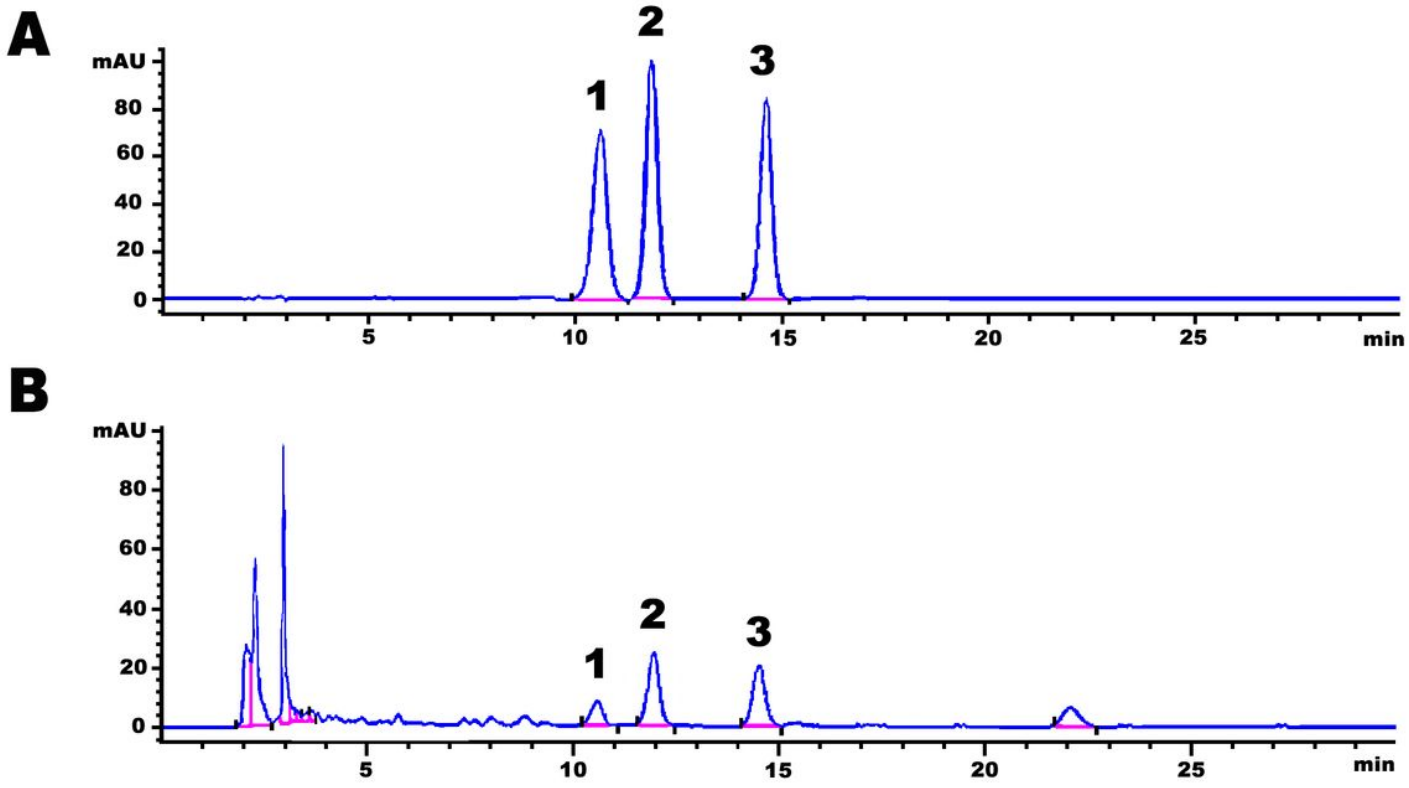

Figure 8

Chromatogram of mixed standard (A) and samples (B). Note: 1. Naringin; 2. Hesperidin; 3. Neohesperidin

Figure 9

Dendrogram of Juhe samples based on genetic diversity, shape and size, and flavonoids

Supplementary Files

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