Int. J. Electrochem. Sci., 15 **(2020)** 9622 – 9630, doi: 10.20964/2020.10.54

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

Phylogenetic Investigation of Yellow Camellias Based on Electrochemical Voltammetric Fingerprints

Yuhong Zheng[1], Jiangwei Zhu[2], Li Fu[3,4] and Qinghua Liu[4]

[1] Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing Botanical Garden, Mem. Sun Yat-Sen, Nanjing 210014, P.R. China
[2] Collaborative Innovation Center of Sustainable Forestry in Southern China of Jiangsu Province, (Nanjing Forestry University), Nanjing 210037, P.R. China
[3] College of Materials and Environmental Engineering, Hangzhou Dianzi University, Hangzhou, 310018, P.R. China
[4] Hongze Fishseeds Biotech, Inc. Hongze, Huaian, 223125, PR China
[*]E-mail: fuli@hdu.edu.cn

Received: 30 June 2020 / *Accepted:* 4 August 2020 / *Published:* 31 August 2020

The electrochemical fingerprints of *Camellia huana, C. tianeensis, C. pubipetala, C. chrysantha, C. nitidissima, C. wumingensis, C. quinqueloculosa, C. tunghinensis, C. euphlebia, C. flavida* and *C. multipetala* were recorded using a screen-printed electrode after leaf tissue modification. The electrochemical fingerprints recorded under phosphate buffer solution and acetic acid buffer solution were derived for species identification. In addition, the intraspecific relationships between these *Camellia spp.* were studied based on the recorded electrochemical profiles. The differences in the electrochemically active compounds in the leaf tissue could reflect the differences at the gene level. These results suggest that *C. pubipetala* has a relatively distant relationship with these species. A previous report claimed that *C. tianeensis* was a variant of *C. huana* and supported the integration of *C. tianeensis* into *C. huana*. The close relationship of *C. tianeensis* and *C. huana* was confirmed by the proposed electrochemical method. *C. multipetala* was first considered to have a close relationship with *C. flavida* because they share many similar features. Later, *C. wumingensis* and *C. quinqueloculosa* were also reported to be highly related to *C. flavida*. Our results suggested that *C. flavida* has a distinct relationship with these three species.

Keywords: Intraspecific relationship; *Camellia*; Polyphyly analysis; Phytochemistry; Leaf extract

1. INTRODUCTION

Yellow camellias have golden yellow petals, and there is important economic value in the cultivation of new varieties of camellia in horticulture, so they have received considerable attention from

botanists [1–3]. More than 40 species of yellow camellias have been reported thus far. However, due to the narrow distribution range and the similar external morphological characteristics of these species, the variations of some species were published as independent species, resulting in a large divergence in the taxonomy of yellow camellias [4–6]. To address the problem of the taxonomy of yellow camellias, many scholars have conducted much research on their morphological characteristics, palynology, karyotype and chromosomes.

The classification and definition of these species are based on traditional morphological characteristics, such as the flower and leaf morphologies and fruit type. In fact, the morphological characteristics of some plants, such as certain shapes of the leaf surface, are susceptible to environmental conditions, and the shapes of the fruits and seeds vary to some extent within and between populations [7–10]. In recent years, the rise of molecular biology technology, especially the development of DNA sequencing technology, has provided an advanced research method for the phylogenetic research of yellow camellias [11–14]. Many scholars have used protein electrophoresis, molecular markers and DNA sequencing to study the classification, genetic relationships and genetic diversity of yellow camellias. These molecular methods commonly produce outcomes different from those of traditional taxonomy. In addition, different molecular methods could give different phylogenetic outcomes. Therefore, the confirmation of phylogenetic results using an alternative method could be used for determining the taxonomic status of species [15].

Chemotaxonomy is a technique used for investigating the phylogenetic position of species based on the differences in the chemical compounds in the plant tissue. However, the traditional chemotaxonomy method suffers some disadvantages, such as that it only tags a few compounds that provide limited genetic information. In addition, plant composition investigation requires the use of expensive instruments with sample pre-treatment. In 2015, a plant phylogenetic study collected the voltammetric fingerprint of plant tissue [16]. The electrochemical curves of the plant tissue give the profile of the electro-active components, which could be used to reflect the phylogenetic position of the species [17]. Our work also showed the possibility of phylogenetic investigation based on electrochemistry taxonomy [18–21].

In this work, the leaf tissue of yellow camellias was used for electrode surface modification. *Camellia huana, C. tianeensis, C. pubipetala, C. chrysantha, C. nitidissima, C. wumingensis, C. quinqueloculosa, C. tunghinensis, C. euphlebia, C. flavida* and *C. multipetala* were deliberately selected for phylogenetic analysis. The electrochemical fingerprint of each species was recorded under two buffer conditions and subsequently used for pattern recognition. Then, the intraspecific relationships of these species were deduced and compared with the polyphyly results deduced from other reports.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

All of the reagents were purchased from Macklin Co., Ltd. and used without purification. Screen-printed electrodes (SPEs) were purchased from Nanjing Youyun Technology Co., Ltd. Phosphate buffer

solution (PBS, 0.1 M pH 7.0) was prepared by mixing stock solutions of 0.1 M disodium hydrogen phosphate and sodium dihydrogen phosphate until the pH reached 7.0. Acetate buffer solution (ABS) was prepared by mixing 0.1 M sodium acetate and acetic acid until the pH reached 4.5.

2.2. Plant leaf collection

Leaves of *C. huana, C. tianeensis, C. pubipetala, C. chrysantha, C. nitidissima, C. wumingensis, C. quinqueloculosa, C. tunghinensis, C. euphlebia, C. flavida and C. multipetala* were collected from the Nanjing Botanic Garden.

2.3. Electrode surface modification

Typically, 0.1 g of thawed plant leaf was ground with 5 mL of water with 2 min of sonication. Then, 5 μL of slurry was dip-coated on an SPE and dried at room temperature.

2.4. Electrochemical fingerprint recording

The electrochemical fingerprint of the plant tissue was recorded using a CHI760E electrochemical workstation. Differential pulse voltammetry (DPV) was used for electrochemical recording.

3. RESULTS AND DISCUSSION

Figure 1 shows a schematic diagram of the fingerprint recording process.

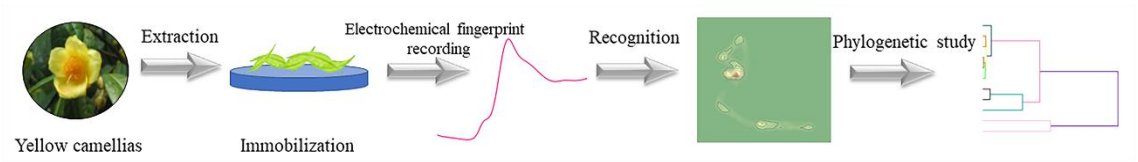

Figure 1. (A) Schematic diagram of recording electrochemical fingerprints for phylogenetic investigation.

Figure 2 and Figure 3 show the DPV curves of the 11 species of yellow camellias recorded under 0.1 M PBS and ABS, respectively. The curves show that all the species yielded oxidation peaks during the scan. These fingerprints represent the information of the electro-active molecules that participate in electrochemical oxidation. Clear differences in the fingerprint can be observed among the species, suggesting that these species present variations in electrochemically active molecules.

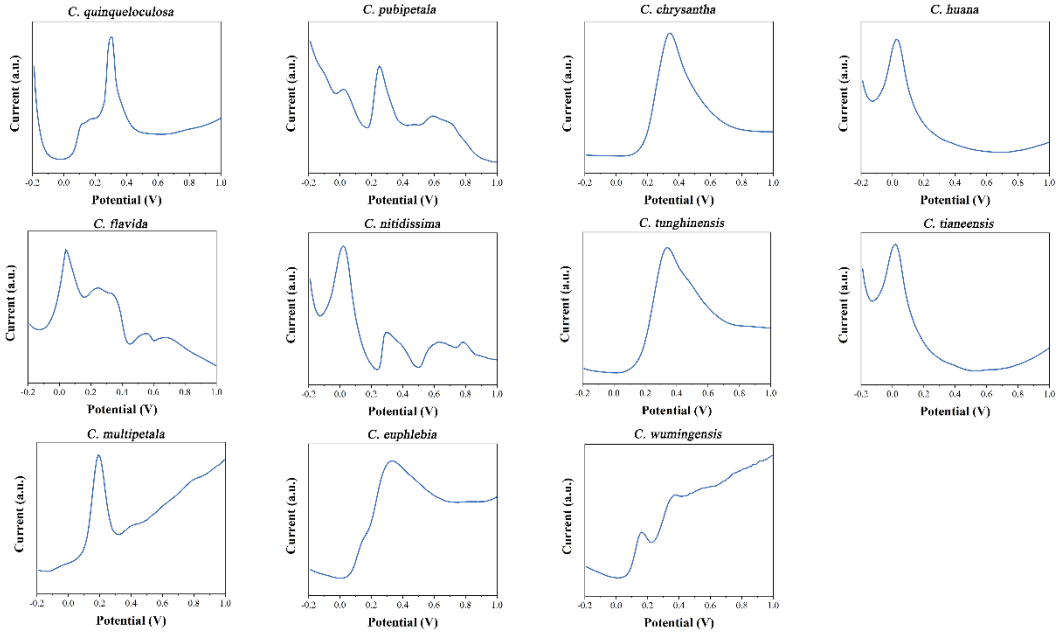

Figure 2. DPV curves of *C. huana*, *C. tianeensis*, *C. pubipetala*, *C. chrysantha*, *C. nitidissima*, *C. wumingensis*, *C. quinqueloculosa*, *C. tunghinensis*, *C. euphlebia*, *C. flavida* and *C. multipetala* recorded on glassy carbon electrodes in 0.1 M PBS.

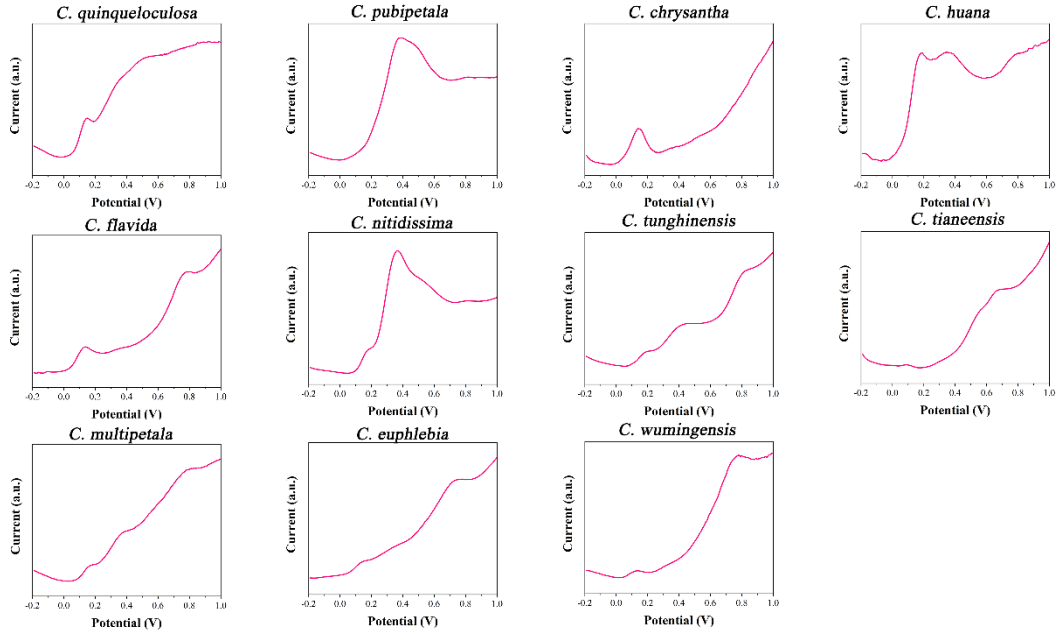

Figure 3. DPV curves of *C. huana*, *C. tianeensis*, *C. pubipetala*, *C. chrysantha*, *C. nitidissima*, *C. wumingensis*, *C. quinqueloculosa*, *C. tunghinensis*, *C. euphlebia*, *C. flavida* and *C. multipetala* recorded on glassy carbon electrodes in 0.1 M ABS.

Species identification using voltammetric curves is not an efficient method. For example, the DPV curves of *C. chrysantha* and *C. tunghinensis* recorded under PBS share some similar characteristics. The DPV curves of *C. euphlebia* and *C. wumingensis* recorded under ABS also look

similar. Therefore, the electrochemical fingerprints recorded from PBS and ABS were used for the construction of the pattern recognition mode. Figure 4 shows 2D density plots of 11 species of yellow camellias using the normalized electrochemical fingerprint from PBS vs. ABS. In this 2D density plot mode, with the assistance of image recognition methods [22], such as voxel similarity measurements [23], unknown species of yellow camellias can be identified.

Figure 5 shows the 3D principal component analysis (PCA) results using the electrochemical data obtained from 11 species of yellow camellias. The three factors extracted within the electrochemical fingerprint reached more than 90% interpretative capability. This result indicated obvious differences in the electrochemically active molecules among these species. As observed in the 3D PCA plot, *C. chrysantha* and *C. multipetala* were closely related, while *C. tianeensis* and *C. huana* were in a group. In addition, *C. pubipetala* and *C. flavida* can be considered outliers among species.

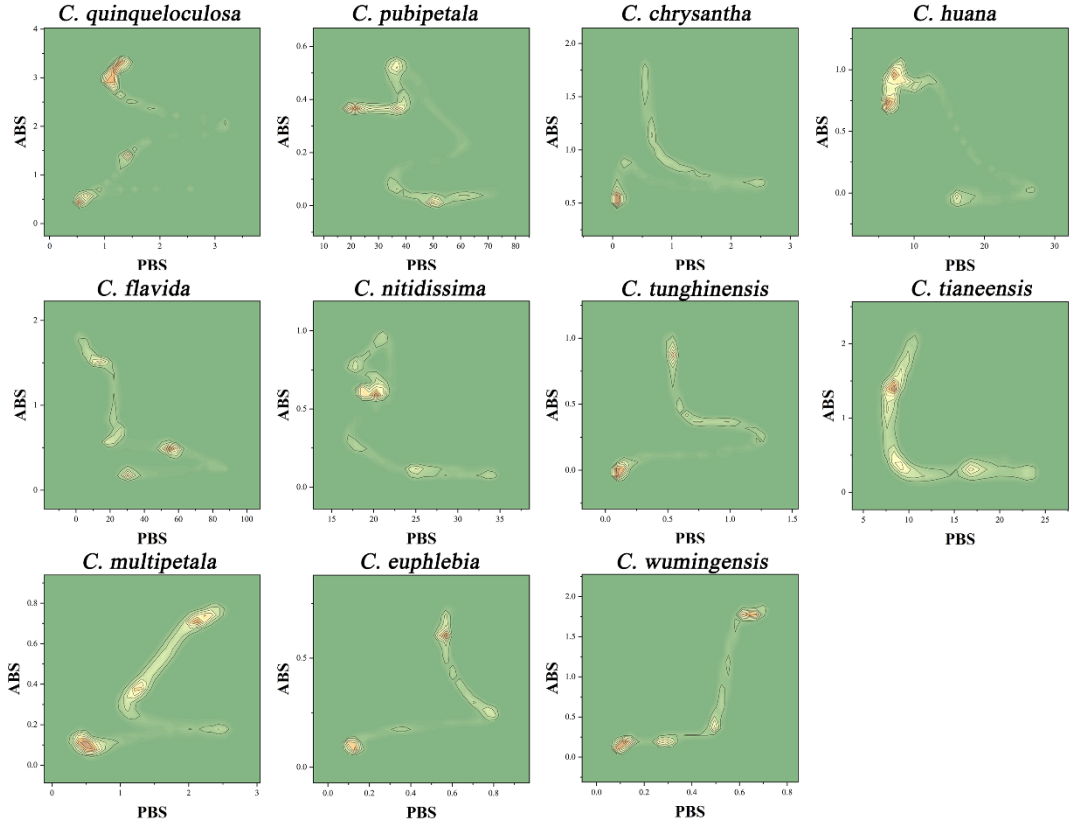

Figure 4. 2D density patterns of *C. huana, C. tianeensis, C. pubipetala, C. chrysantha, C. nitidissima, C. wumingensis, C. quinqueloculosa, C. tunghinensis, C. euphlebia, C. flavida* and *C. multipetala* based on normalized fingerprints recorded from 0.1 M PBS vs. 0.1 M ABS.

Figure 6 shows the dendrogram of *C. huana, C. tianeensis, C. pubipetala, C. chrysantha, C. nitidissima, C. wumingensis, C. quinqueloculosa, C. tunghinensis, C. euphlebia, C. flavida* and *C. multipetala* deduced from the electrochemical fingerprints recorded in two buffer solutions. The phylogenetic tree was divided into four main clades. The first clade included *C. quinqueloculosa, C.*

chrysantha, and *C. multipetala*. The second clade included *C. tunghinensis*, *C. euphlebia* and *C. wumingensis*. The third group included *C. huana*, *C. tianeensis* and *C. nitidissima*. The last clade included *C. pubipetala* and *C. flavida*. The PC1, PC2 and PC3 factors extracted within the voltammetric data could reach more than 90% interpretative capability, suggesting that there were significant differences among the electrochemical profiles of the samples studied in this work.

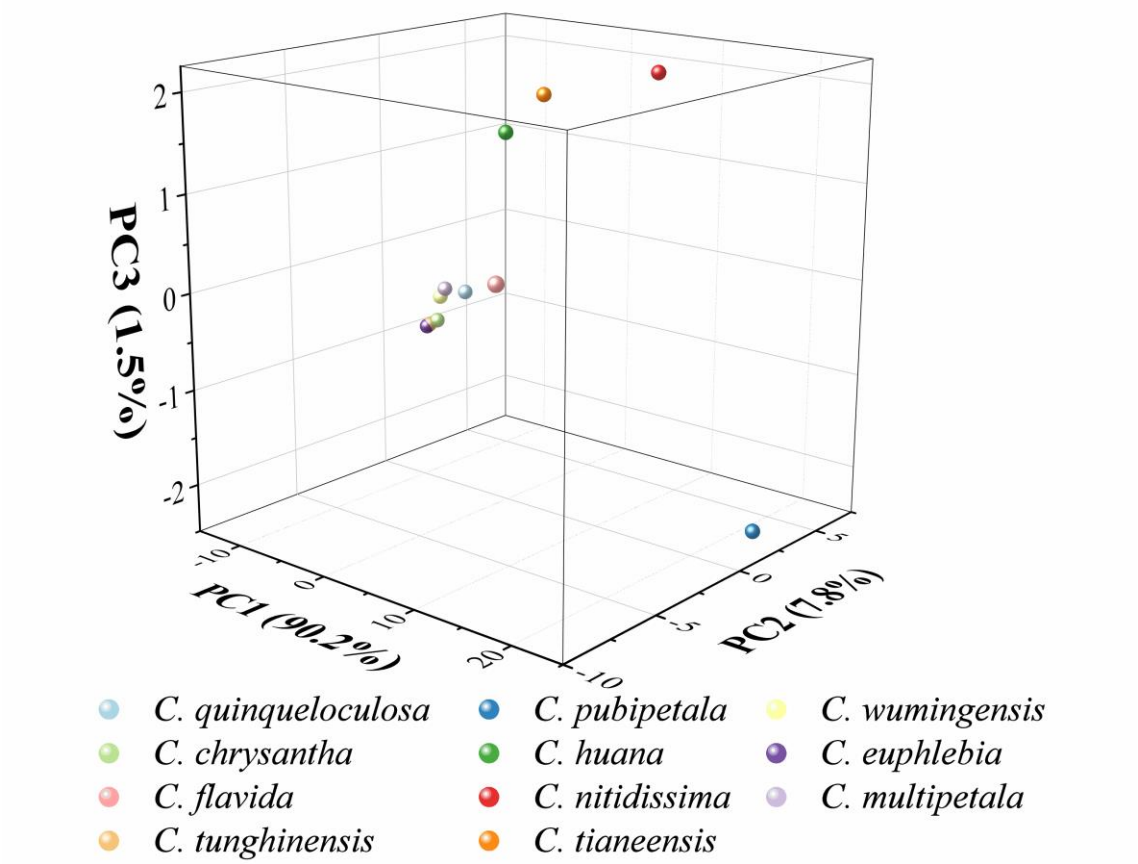

Figure 5. 3D PCA analysis of *C. huana, C. tianeensis, C. pubipetala, C. chrysantha, C. nitidissima, C. wumingensis, C. quinqueloculosa, C. tunghinensis, C. euphlebia, C. flavida* and *C. multipetala* based on normalized fingerprints recorded from 0.1 M PBS and 0.1 M ABS.

Plant leaf tissue was ground and sonicated with the assistance of water to achieve fast extraction. The immobilization of the plant tissue on a glassy carbon electrode was carried out at room temperature. Then, the electrochemical fingerprint was recorded using a DPV scan under PBS or ABS conditions. Two sets of electrochemical fingerprints were then used to generate the 2D density pattern. This pattern can be used for species identification since direct species identification using the voltammetric curve is not an efficient method. Then, the phylogenetic tree of these species was deduced based on the electrochemical fingerprints for polyphyly analysis.

The oxidation peaks observed during the voltammetric scan indicated that some electroactive compounds were oxidized. Previous studies have reported that polyphenols, flavonoids and alkaloids in plant tissues can be oxidized at low potentials [24–28].

As shown in the 2D density plots, each species of yellow camellia showed a different pattern. As we mentioned in the Results section, *C. chrysantha* and *C. tunghinensis* showed similar DPV curves under PBS, probably because they have similar electro-active compounds that participate in electrochemical oxidation. In contrast, the 2D density plots of *C. chrysantha* and *C. tunghinensis* showed a very large difference, suggesting that the pattern recognition mode is a more effective method for plant species identification.

Some reports have conducted studies on the phylogenetic relationship of yellow camellias using RAPD, ISSR, AFLP, and nuclear gene ITS sequences. However, molecular markers are generally used in population genetics research. The ITS sequence of Camellia plants is a multi-copy, high-mutation region with insertions or deletions between tandem repeat units. Therefore, the results of these studies are highly controversial. Although the environment affects the chemical compound distribution in plant species, genes are still the most significant factor. As shown in Figure 6, *C. pubipetala* showed a relatively distant relationship with these species. This result is confirmed by other studies since the branches and leaves of *C. pubipetala* are covered with fluff, which is quite different from other species [29,30]. A previous report claimed that *C. tianeensis* was a variant of *C. huana* and supported the integration of *C. tianeensis* into *C. huana* [31,32]. Our results strongly support the close relationship between *C. tianeensis* and *C. huana*. *C. multipetala* was first considered to have a close relationship with *C. flavida* due to the high similarity of their morphological features [33]. Later, *C. wumingensis* and *C. quinqueloculosa* were also reported to be highly related to *C. flavida* [34]. Our results suggested that *C. flavida* has a distinct relationship with the other three species. However, *C. wumingensis*, *C. quinqueloculosa* and *C. multipetala* were closely related.

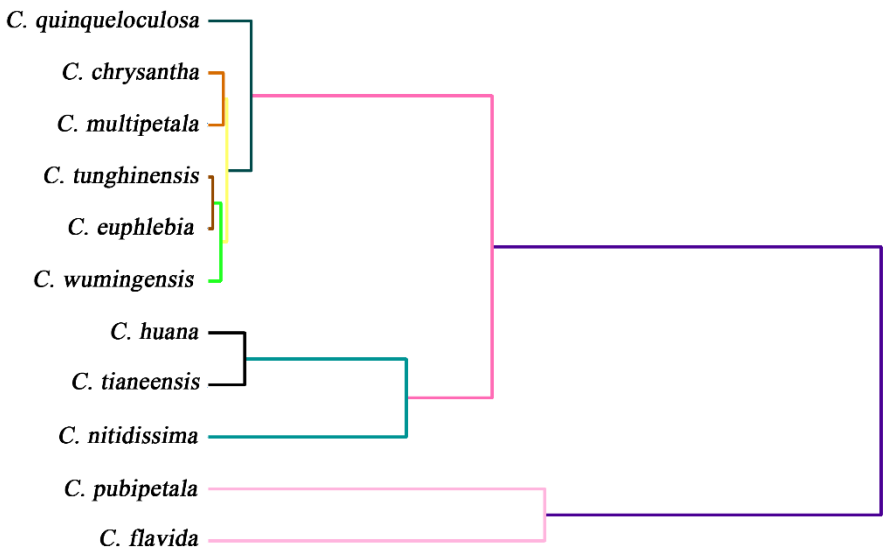

Figure 6. Dendrogram of *C. huana, C. tianeensis, C. pubipetala, C. chrysantha, C. nitidissima, C. wumingensis, C. quinqueloculosa, C. tunghinensis, C. euphlebia, C. flavida* and *C. multipetala* based on the electrochemical fingerprint recorded under two buffer solutions.

4. CONCLUSION

In conclusion, the electrochemical fingerprints of *C. huana, C. tianeensis, C. pubipetala, C. chrysantha, C. nitidissima, C. wumingensis, C. quinqueloculosa, C. tunghinensis, C. euphlebia, C. flavida* and *C. multipetala* were used for species identification and phylogenetic study. The fingerprints were successfully recorded under PBS and ABS conditions. These fingerprints were used for 2D density plot construction and species identification. The dendrogram obtained from the electrochemical fingerprints yields a persuasive phylogenetic result compared with those of other investigations. The dendrogram indicates the following:

(1) *C. pubipetala* showed a relative distant relationship with the other species.

(2) Our results strongly supported the close relationship between *C. tianeensis* and *C. huana*.

(3) Our results suggested that *C. flavida* has a distinct relationship with *C. wumingensis*, *C. quinqueloculosa* and *C. multipetala*. However, *C. wumingensis, C. quinqueloculosa* and *C. multipetala* are closely related.

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