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Electroanalytical study of infrageneric relationship of *Lagerstroemia* using glassy carbon electrode recorded voltammograms

Estudio electroanalítico de la relación infragenérica de *Lagerstroemia* utilizando voltamogramas registrados con electrodo de vidrio de carbono

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Abstract

An electrochemical fingerprint recording process has been developed for plant species identification and infrageneric relationship analysis. Electrochemical profiles of *Lagerstroemia caudata, L. ambigua, L. speciosa, L. anhuiensis, L. balansae, L. minuticarpa, L. subcostata, L. indicas, L. fauriei, L. guilinensis, L. limii, Heimia myrtifolia* and Lawsonia inermis were recorded using leaf extract under three buffer solutions. The voltammetric data recorded under different buffer solutions can be derived as a scatter plot and a 2D density pattern for species recognition. As the distribution of electrochemical active compounds in plants is controlled by genes, these fingerprints can reflect differences at the genetic level between species. The dendrogram deduced from the electrochemical fingerprint has been used as evidence for infrageneric relationship investigation. The dendrogram deduced from electrochemical fingerprint indicates the Heimia myrtifolia and Lawsonia inermis were separated from other species, which is in a good agreement with their exotaxa position in this investigation. The intergeneric relationship deduced from data suggested the L. speciosa and L. indicas in a distant relationship.

Keywords: Electrochemical fingerprints; species identification; taxonomic sensing; solid state electrochemistry; *Lagerstroemia*.

Resumen

Se ha desarrollado un proceso de registro electroquímico de huellas dactilares para la identificación de especies de plantas y el análisis de relaciones infragenéricas. Perfiles electroquímicos de Lagerstroemia caudata, *L. ambigua, L. speciosa, L. anhuiensis, L. balansae, L. minuticarpa, L. subcostata, L. indicas, L. fauriei, L. guilinensis, L. limii, Heimia myrtifolia* y *Lawsonia inermis* se registraron utilizando extracto de hoja en tres soluciones tampón. Los datos voltamétricos registrados bajo diferentes soluciones tampón se pueden derivar como un diagrama de dispersión y un patrón de densidad 2D para el reconocimiento de especies. Como la distribución de los compuestos electroquímicos activos en las plantas está controlada por genes, estas huellas digitales pueden reflejar diferencias a nivel genético entre especies. El dendrograma deducido de la huella digital electroquímica se ha utilizado como evidencia para la investigación de la relación infragenérica. El dendrograma deducido de la huella digital electroquímica indica que Heimia myrtifolia y Lawsonia inermis se separaron de otras especies, lo que concuerda con su posición de exotaxa en esta investigación. La relación intergenérica deducida de los datos sugirió a L. speciosa y L. indicas en una relación distante.

Palabras clave: Huellas electroquímicas; identificación de especies; detección taxonómica; electroquímica en estado sólido; *Lagerstroemia*.

1 Introduction

Lagerstroemia is a genus contains more than 50 species belong to Lythraceae distributed in the eastern, southeastern and southern subtropical regions of Asia, the island of New Guinea, the Philippines and Australia (Xu *et al*., 2017). Most of the species have large and beautiful flowers, which have high ornamental value and are commonly used as ornamental trees. Some species can grow into trees in limestone mountain which is an excellent candidate for mountain environment protection (Adhikari *et al*., 2019). Some of these species are hard wood with strong resistance to termite, therefore, it is precious wood used for interior decoration, shipbuilding, architecture and furniture.

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Their cultivation has a history of at least 1500 years in China (Amresh *et al*., 2018). The phylogenetic relationship within *Lagerstroemia* has been studied using either morphological way or molecular-based techniques (such as *rbc*L gene, *trn*L-F region, ITS etc.) (Graham *et al*., 2005; Huang and Shi, 2002; Suo *et al*., 2015, 2012; Xiang *et al*., 2011; Zl *et al*., 2016). However, due to many shared morphological features, genetic diversity cannot be fully explained using the traditional morphological analysis. On the other hand, molecular studies also revealed some controversial results due to the selection of different DNA markers. This is a very common phenomenon in plant phylogenetic studies. Therefore, many alternative analytical methods, such as cytotaxonomy (Almeida *et al*., 2017; de Moraes *et al*., 2017), karyosystematics (Barbosa *et al*., 2017; Di-Nizo *et al*., 2017), chemotaxonomy (Wink *et al*., 2018; Xu *et al*., 2016) and palynology (McGlone *et al*., 2017; Sinopoli *et al*., 2018). have been developed for providing evidence of the phylogenetic status of species.

Among these methods, chemotaxonomy is a biological classification method based on similarities in certain compounds among the organisms. The difference between the target compounds has been used as indicators of genetic relationships because of plant species with close relatives often have similar physiological and biochemical characteristics due to their close genetic relationship. For example, Mori *et al*. (Mori *et al*., 2018) used fatty acids as indicators for investigating the phylogenetic relationship within family Selenastraceae. Negrin *et al*. (Negrin *et al*., 2019) recently used chemotaxonomy for investigating some caffeine-containing species in genus *Ilex* and their phylogenetic relationship within family Aquifoliaceae. Chromatography, mass spectrum and NMR are the main techniques used for the analysis of chemical components in the plant. The target molecules have been further used for chemotaxonomy purposes. However, these instruments need a sophisticated operation process with long-time analysis. On the other hand, traditional chemotaxonomy focuses on one type of chemical compound during the analysis, which reflects insufficient evidence for genetic difference. Therefore, chemotaxonomy is not a preferred method for plant phylogenetic analysis.

Electroanalysis is a widely studied analytical technique for the detection and identification of analytes *in vivo* or *in vitro* (Bijad *et al*., 2013, 2018; Fu *et al*., 2020a; Karimi-Maleh and Arotiba, 2020; Wu *et al*., 2017; Ying *et al*., 2020). Using a range of signal amplification techniques, electrochemical sensors can detect very low concentrations of the target analyte. So far, it has been widely used for environmental pollutant monitoring (Karimi-Maleh *et al*., 2019, 2020b), pharmaceutical compound detection (Karimi-Maleh *et al*., 2020a; Khodadadi *et al*., 2019) and food analysis (Shamsadin-Azad *et al*., 2019; Tahernejad-Javazmi *et al*., 2019). In addition to the detection of a single substance, electroanalytical techniques can also scan for electrochemical active compounds in complex systems (Fu *et al*., 2020b; Li *et al*., 2019; Zhang *et al*., 2019). It has been used for plant identification based on the fingerprinting of electrochemical active compounds in plant tissue (Doménech-Carbó *et al*., 2015a; Domínguez and Doménech-Carbó, 2015). Later on, these fingerprint has been correlated to the phylogenetic position of the plant species and showed convincing results (Doménech-Carbó *et al*., 2017, 2015b; Doménech-Carbó *et al*., 2017). Our previous works also demonstrated the feasibility of the electroanalysis of plant tissue to the phylogenetic position of the plant (Fu *et al*., 2019a, 2019b, 2018a, 2018b). Species from *Lycoris* have been selected as samples for species identification and infrageneric relationship investigation. In this study, we extend the methodology towards species of *Lagerstroemia*. 11 species of *Lagerstroemia* with two species as exotaxa were selected deliberately. We also used an advanced electrochemical fingerprint recording process for improving accuracy. In addition, for faster identification of species, we proposed a new pattern recognition in this work.

2 Materials and methods

Leaves of *Lagerstroemia caudata, L. ambigua, L. speciosa, L. anhuiensis, L. balansae, L. minuticarpa, L. subcostata, L. indicas, L. fauriei, L. guilinensis, L. limii, Heimia myrtifolia* and *Lawsonia inermis* were collected from Nanjing Botanical Garden Mem. Sun Yat-Sen (Nanjing, China) in September 2019. Healthy leaves of each species were carefully collected and stored at -20 ℃ before analysis. KH_2PO_4, Na_2HPO_4, sodium acetate, acetic acid and Tris powder were purchased from Macklin Co., Ltd. All other chemicals were analytical-grade reagents and were used without further purification. The reference electrode (Ag/AgCl), counter electrode (Pt wire) and working electrode (glassy carbon electrode, 3 mm in diameter, GCE) all purchased from Gaoshi Ruilian

Co.Ltd. (Wuhan, Chian) Milli-Q water (18.2 MΩ/cm) was used throughout the experiments.

0.1 M acetate buffer (ABS, pH 4.5), phosphate buffer (PBS, pH 7), or Tris buffer (Tris, pH 9.0) was directly used as a solvent for plant leaf extract preparation. Typically, 10 mL of buffer solution was added into 2 g chopped plant leaf by 1 min grinding. The slurry was sonicated using a bath sonicator for 5 min. The supernatant was then transferred to a new beaker used as analyte. For electrochemical fingerprint recording, a GCE was polished with an alumina-water slurry and rinsed with ethanol and water. Then, a three-electrode system was inserted into the beaker for electrochemical fingerprint recording. All electrochemical fingerprints were recorded using a CHI660C working station. Differential pulse voltammetry was used for recording the electrochemical fingerprints of all plant tissue between 0-1.4 V with a pulse amplitude of 50 mV, pulse width of 0.05 s and pulse period of 0.5 s.

3 Results and discussion

Figure 1 shows a schematic diagram of the proposed electrochemical fingerprint recording process. A simple leaf tissue extraction process was carried out using different buffer solutions directly to avoiding the solvent elimination process before the electrochemical fingerprint recording. Moreover, the direct recording electrochemical fingerprint using extract showed more reproducible results compared with that of the data recorded using plant tissue modification method (Fu *et al.*, 2019a, 2019b, 2018a, 2018b). Normally, leaf tissue contains a large number of long-chain molecules of cellulose with poor electroconductivity. The elimination of their effect could provide a more comprehensive electrochemical profile of the electro-active compounds in leaf. Figure 2 shows DPV curves of 11 species of *Lagerstroemia* with two exotaxa species recorded using 0.1 M PBS as the extraction solvent and electrolyte (DPV curves of each species recorded in 0.1 ABS and Tris are shown in Figure S1 and S2). It is pertinent to note that each species showed several peaks between 0 V and 1.4 V correspond to the oxidation of electro-active compounds. These compounds could be flavonols (Liu *et al.*, 2020), phenolic acids (Irakli *et al.*, 2018), procyanidins (Ramsay and Mueller-Harvey, 2016), alkaloids (Marín-Sáez *et al.*, 2019) and pigments (Ayranci *et al.*, 2019; Calimli *et al.*, 2020; Chou *et al.*, 2020; Şavk *et al.*, 2019; Şavk *et al.*, 2020). In the case of quercetin, the main oxidation process consists of the two-electron, two proton oxidation of the 3'4'-dihydroxybenzoic acid moiety yielding the corresponding o-quinone. The principle of the electro-chemotaxonomy is based on the statistical analysis of the difference of the whole profile rather than the quantitative detection of a particular compound. Therefore, we do not need to identify each oxidation peak recorded during the DPV scan.

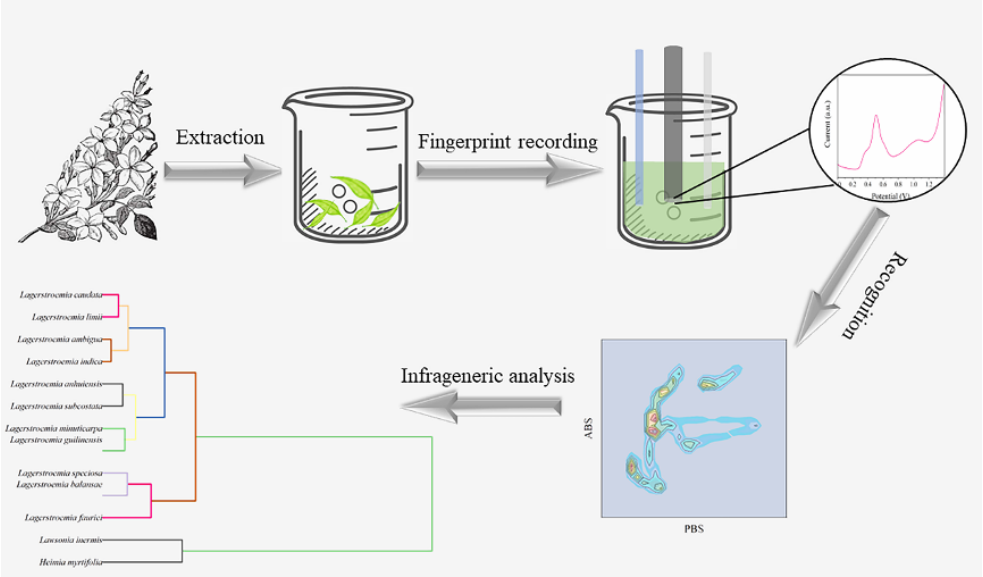

Fig. 1. Schematic diagram of electrochemical biosensor for plant fingerprint recording.

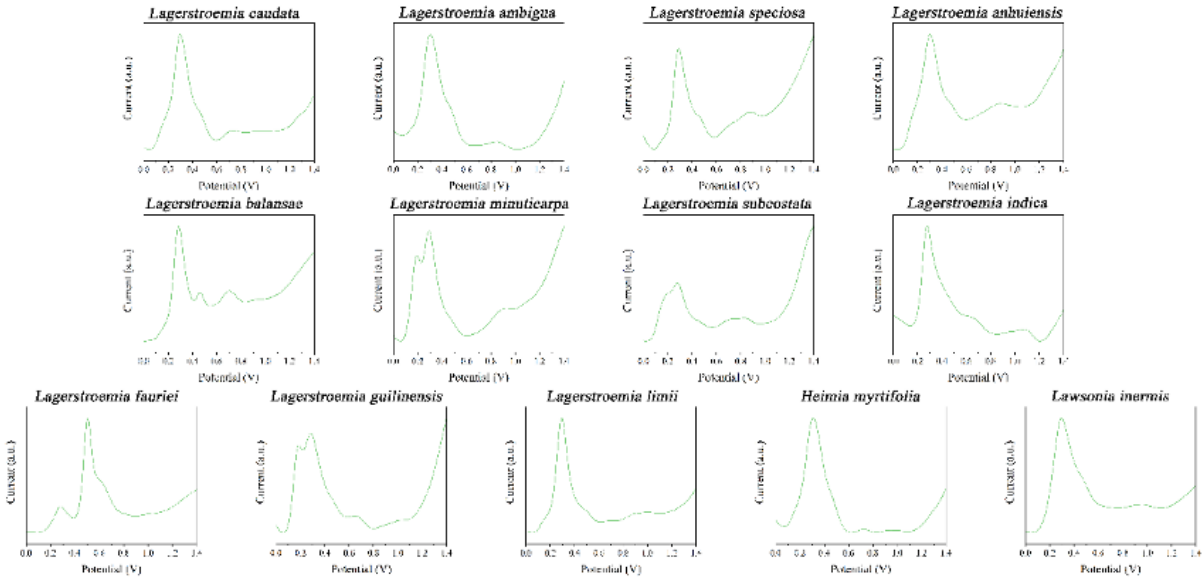

Fig. 2. DPV curves of 13 species recorded in 0.1 M PBS. Additional DPV curves of each species recorded in 0.1 ABS and Tris are shown in Figure S1 and S2.

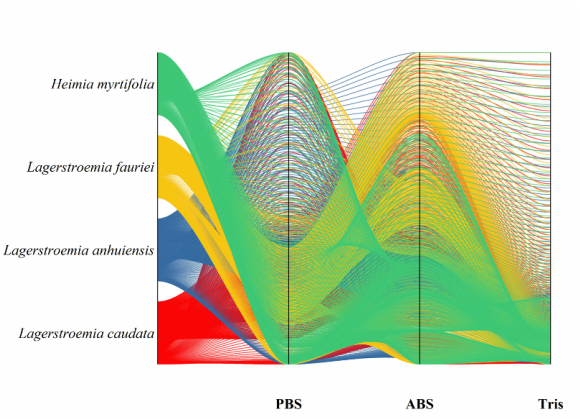

Fig. 3. Parallel coordinate plot of normalized currents of *L. caudata, L. ambigua, L. speciosa, L. anhuiensis* and *L. balansae* recorded in 0.1 M PBS, ABS and Tris.

In fact, for a complex system like plant leaf, the direct electrochemical recording has many limitations for specific compound analysis. However, the total voltammetric profile contains all information of responded oxidizable compounds. Therefore, it can be considered as the profile of electrochemical active compounds from plant tissue participated in electrochemical oxidation under buffer condition. As shown in the Figure 2, as well as Figure S1 and S2, it is pertinent to note that the obvious differences of the electrochemical fingerprint can be noticed between species, indicating the variation of the oxidized compounds between species. The parallel coordinate plot is a common method to analyze and display multivariate data. We used it for evaluating whether a clear difference of electrochemical profile can be noticed between the species. Figure 3 shows the parallel coordinate plot of the normalized current of *L. caudata, L. anhuiensis, L. fauriei* and *Heimia myrtifolia* recorded in 0.1 M PBS, ABS and Tris as examples. As shown in the figure, each species showed different tendencies, suggesting different species show discernible differences recorded in the different buffer solutions. Based on this observation, the electrochemical fingerprint recorded from leaf extract showed the potential for species recognition. The rapid identification of plant species is important in agriculture, horticulture and plant-based medical materials production(Hernández-Carrillo *et al*., 2019; Marques *et al*., 2019; Ruiz-Palomino *et al*., 2019). It is difficult to identify them only by morphological features. In this case, the difference of the electrochemical fingerprints recorded for each species can be used for pattern generation and subsequently used for rapid identification.

The identification of species using DPV curves is not an efficient way. The electrochemical fingerprints of some of these species share some similar characteristics, such as *L. speciosa* and *L. anhuiensis* (Figure 2). In contrast, pattern identification based on multiple electrochemical fingerprints showed a more effective performance.

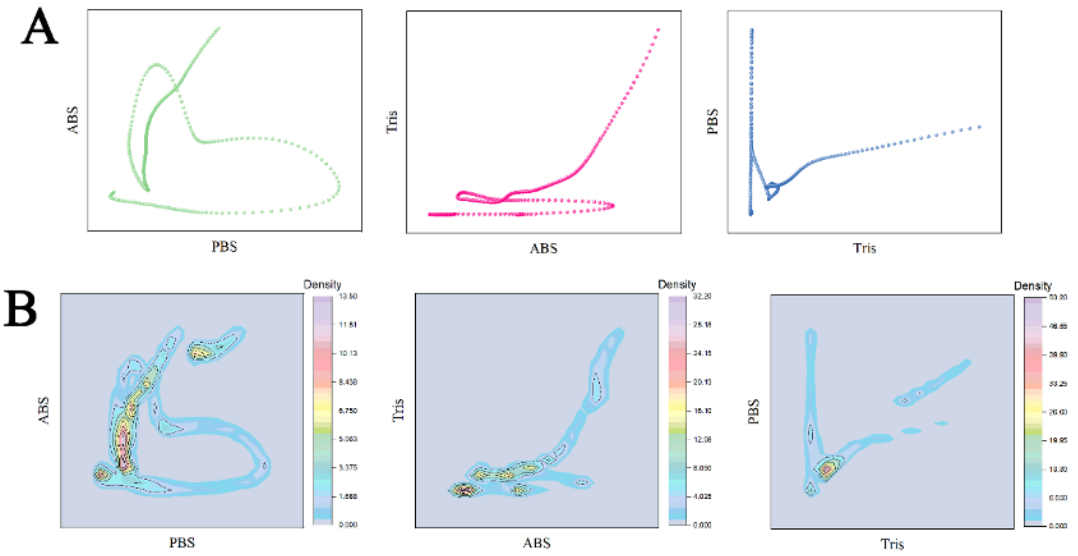

Fig. 4. (A) 2D scatter patterns and (B) 2D density patterns of *L. caudata* based on normalized fingerprint recorded from 0.1 M PBS vs. 0.1 M ABS, 0.1 M ABS vs. 0.1 M Tris and 0.1 M Tris vs. 0.1 M PBS. Additional 2D scatter patterns and 2D density patterns of each species are shown in Figure S3-S14.

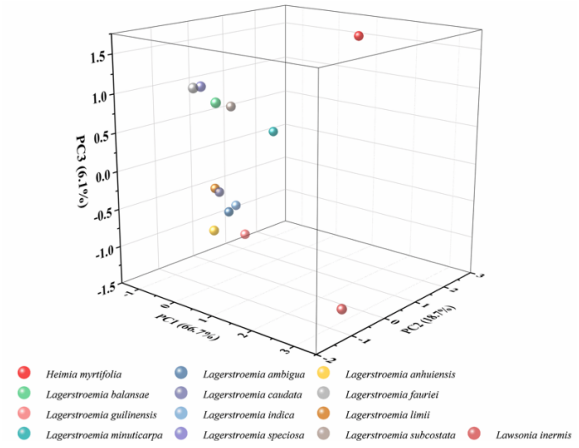

Fig. 5 3D PCA analysis of *L. caudata, L. ambigua, L. speciosa, L. anhuiensis, L. balansae, L. minuticarpa, L. subcostata, L. indicas, L. fauriei, L. guilinensis, L. limii, Heimia myrtifolia* and *Lawsonia inermis* using normalized current recorded after three solvents extractions.

Figure 4A shows a series of scatter patterns of *L. caudata* deduced from the fingerprint recorded from three buffer solution (Figure S3A-14A show the scatter patterns of *L. ambigua, L. speciosa, L. anhuiensis, L. balansae, L. minuticarpa, L. subcostata, L. indicas, L. fauriei, L. guilinensis, L. limii, Heimia myrtifolia* and *Lawsonia inermis*, respectively). The figure clearly shows that the scatter pattern is easier to identify species than the direct electrochemical fingerprint. However, the recognition of species based on scatter pattern require region segmentation and point calculation, which still not achieve fast visual recognition. In this work, we further proposed a 2D density pattern for species identification. Figure 4B shows the 2D density patterns of *L. caudata* deduced from same data used for scatter plots. Figure S3B-14B show the 2D density patterns of *L. ambigua, L. speciosa, L. anhuiensis, L. balansae, L. minuticarpa, L. subcostata, L. indicas, L. fauriei, L. guilinensis, L. limii, Heimia myrtifolia* and *Lawsonia inermis*, respectively. In this recognition model with the assistance of well-established image recognition methods (A. K. Jain *et al*., 2000), such as voxel similarity measurement and image segmentation-based similarity measurement (M. Holden *et al*., 2000), unknown species can be identified by matching patterns or even by locating high-density areas with the database.

In order to further testing the difference of electrochemical profile between the species, the principal component analysis (PCA) analysis has been carried out.

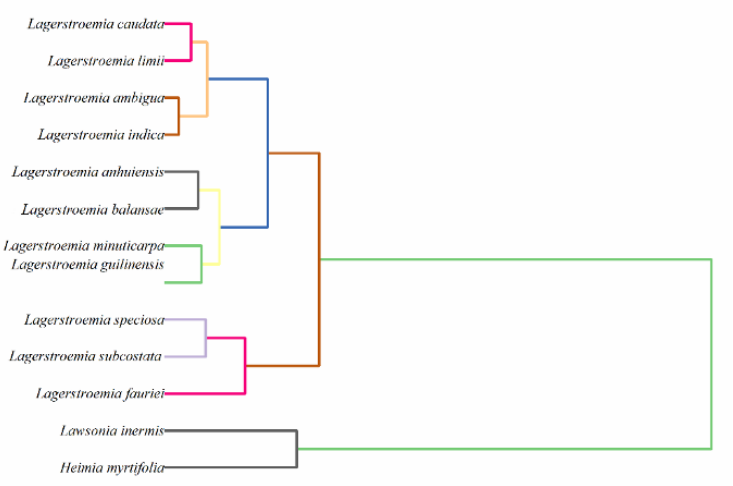

Fig. 6. Dendrogram of *L. caudata, L. ambigua, L. speciosa, L. anhuiensis, L. balansae, L. minuticarpa, L. subcostata, L. indicas, L. fauriei, L. guilinensis, L. limii, Heimia myrtifolia* and *Lawsonia inermis* based on the electrochemical fingerprints recorded in three buffer solutions.

Our previous studies indicated that the PCA result of the electrochemical profile does not have a high interpretative capability (Fu *et al*., 2019a, 2019b). In this case, as shown in Figure 5, the three factors extracted within the voltammetric data can reach more than 90% interpretative capability, suggesting there were significant differences in electrochemical profiles among the species. The 3D PCA grouping result showed *L. limii, L. caudata, L. ambigua, L. indica, L. anhuiensis* and *L. guilinensis* were grouped in a close spatial position. *L. fauriei, L.speciosa, L. balansae* and *C. latifolium* were grouped in a close spatial position. In addition, *Heimia myrtifolia* and *Lawsonia inermis* can be considered as outliers among the species. Although ecology has a great influence on the type and distribution of chemicals in plant tissues, genes are still the most important factor. The results indicate the obvious differences in electro-active compounds among species, reflecting that there may also be significant differences at the gene level.

Since the electrochemical fingerprint of species is positively correlated with the distribution and amount of electro-active compounds, we attempted to use the above-mentioned fingerprint for dendrogram analysis. Figure 6 shows the dendrogram of 11 species of *Lagerstroemia* with two exotaxa species deduced from the electrochemical fingerprint recorded using three buffer solutions. The phylogenetic tree was divided into four main principal infrageneric clades. The first clade consisted of the species *L. caudata, L. limii, L. ambigua* and *L. indica*. The second clade consisted of the species *L. anhuiensis, L. balansae, L. minuticarpa* and *L. guilinensis*. The third group consisted of the species *L. speciosa, L. subcostata and L. faurieia*. The last clade consisted of the species *Heimia myrtifolia* and *Lawsonia inermis*. The intergeneric relationships within the *Lagerstroemia* are a very complex topic that faces many challenges even using molecular sequencing (Xu *et al*., 2017). The dendrogram deduced from electrochemical fingerprint indicates the *Heimia myrtifolia* and *Lawsonia inermis* were separated from other species, which is in a good agreement with their exotaxa position in this investigation. L. speciosa is popular in horticulture, however, the species delimitation still faces challenges. The intergeneric relationship deduced from complete chloroplast genomes suggested the *L. speciosa* and *L. indicas* in a close relationship (Xu *et al*., 2017; Zheng *et al*., 2020), while the ITS sequence result suggested they have a distant relationship (Nurcahyanti *et al*., 2018). Our results endorsed the finding of the latter. The intergeneric relationship between *L. guilinensis* and *L. fauriei* deduced from the complete chloroplast genome is very similar to our result (Gu *et al*., 2017). The dendrogram deduced from electrochemical fingerprint *L. subscostata* and *L. fauriei* have a close relationship, which confirmed with the chloroplast genome sequence (Khan *et al*., 2018). We believe these results provided valuable evidence to the phylogenetic assumption proposed by botanist.

Conclusions

In conclusion, the electrochemical fingerprint of *L. caudata, L. ambigua, L. speciosa, L. anhuiensis, L. balansae, L. minuticarpa, L. subcostata, L. indicas, L. fauriei, L. guilinensis, L. limii, Heimia myrtifolia* and *Lawsonia inermis* were recorded by a glassy carbon electrode using plant leaf extract. Based on the recorded electrochemical fingerprint, these species can be effectively identified using pattern recognition based on the 2D density plot. In addition, the electrochemical fingerprints have been used for phylogenetic analysis. The dendrogram deduced from the electrochemical fingerprints of 11 species of *Lagerstroemia* with two exotaxa species gave a persuasive taxonomical result compared with molecular studies. The dendrogram deduced from electrochemical fingerprint indicates the *Heimia myrtifolia* and *Lawsonia inermis* were separated from other species, which is in a good agreement with their exotaxa position in this investigation. The intergeneric relationship deduced from data suggested the *L. speciosa* and *L. indicas* in a distant relationship. *L. subscostata* and *L. fauriei* have a close relationship.

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