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Chemical composition of floral scents from three *Plumeria rubra* L. (Apocynaceae) forms linked to petal color proprieties

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ABSTRACT

The floral scents of three forms of cultivated *Plumeria rubra* L. were evaluated through mass flowering phenology using the dynamic headspace adsorption method and were identified with coupled gas chromatography and mass spectrometry. The forms *P. rubra* f. *acutifolia* and *P. rubra* f. *lutea* had white and yellow flower petals, respectively, and the flower petals of *P. rubra* f. *rubra* were red. Although 68 components of the flower scents of the three forms were recorded in different proportions, only 14 chemical compounds were identified with statistically significance. The main volatile compounds in the red form of *P. rubra* L. were fatty acid derivatives (56.75%). The main compounds in the white and yellow forms of *P. rubra* L. were benzenoid and terpene, with proportions of 48.38% and 33.33% in *P. rubra* f. *acutifolia* and proportions of 42.30% and 47.43% in *P. rubra* f. *lutea*, respectively. These differences in the flower scents might be one result of the minor genetic differences between these forms, similar to the role of genetic differences in the flower color combinations of the three forms. We conclude that petal color traits can, to some extent, reflect differences in floral scent compositions and that minor genetic differences of different plant forms exert impacts not only on flower color but also on the phytochemistry of floral scents.

1. Introduction

Plumeria rubra L. is a typical tropical plant species that is originally native to Central and South America, including Mexico, Colombia and Venezuela. *P. rubra* L. has been widely cultivated in subtropical and tropical climates worldwide and is a popular garden and park plant because of its graceful morphology and refreshing aromatics. *P. rubra* L. has many different color morphs with mass-flowering phenology and is composed of fragrant flowers of shades of pink, white and yellow throughout the summer and autumn (Stephen, 2008). Moreover, *P. rubra* L. has been defined as a deceptive pollination species that does not provide any nectar or food rewards, although it shares many traits for hawk moth-pollinated plants (Haber, 1984). The mass-flowering phenomenon not only occurred in *Plumeria* species with large infundibuliform flowers and tiny inflorescence bracts but also in some other families, such as, Araceae, Orchidaceae, and Scrophulariaceae (Ackerman, 1986; Haber, 1984; Papadopulos et al., 2013). The specific volatile components of floral scents were believed to be the major vectors to deceptively attract pollination agents.

Undoubtedly, floral color and scent are the most important attractants for pollinators (Chen et al., 2012; Knudsen and Tollsten, 1993; Veiga et al., 2015). Such combinations have been documented among individuals of the same or different deceptive plant species (Dafni, 1984; Salzmann and Schiestl, 2007; Tollsten and Bergström, 1993). A meta-analysis showed that the flower scent components and color display specificity of deceptive-pollinated species are more variable than in species offering a reward to pollinators (Ackerman et al., 2011). The variation of the combination between floral scent and color may be closely associated with distinct pollinator species and foraging preferences. Previous studies have given rise to the hypothesis that white color with aromatic compounds (benzenoids), particularly alcohols and esters, were particularly preferred by nocturnal hawk moths (Majetic et al., 2007, 2008; Raguso et al., 2003). More recent results showed that flower color and scent are intrinsically coupled, and the evolutionary

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implications are complex (Bischoff et al., 2015; Policha et al., 2016).

Therefore, intra-specific floral polymorphisms provide an opportunity to interpret the distinctive selection behavior of pollination agents (Dormont et al., 2010; Gong et al., 2014). For example, hawk moth and hummingbird pollinators impose divergent selection pressure on yellow and red morphs of *Mimulus* (Scrophulariaceae) (Streisfeld and Kohn, 2007). However, a change in flower color or a lack of flower color is a frequent and universal fact in wild populations, and it is also produced in cultivated varieties or ornamental species by human domestication of the species (Chang, 2016; Tooke and Battey, 2010). The diverse combinations of flower scent and color occur not only in natural populations (Brown and Clegg, 1984; M. Eckhart et al., 2006; Waser and Price, 1981) but also in cultivated individuals through human hybridization activities, whether deliberate or not. The high polymorphic floral traits reflect genetic assemblage or phenotypic plasticity, and these lead to the final population structures.

Many studies have emphasized the existence of variations in scent components with distinct color morphs (Li et al., 2006; Majetic et al., 2007; Odell et al., 1999; Salzmann and Schiestl, 2007). Color-scent relationships are complex and diverse, and they largely rely on shared biosynthetic pathways (Armbruster, 2002; Majetic et al., 2007). This fact means that any mutation in a gene coding for an enzyme or a regulatory element will have an impact both on color and scent emission. For example, Zuker et al. (2002) found that the suppression of a single key enzyme led to the formation of white-flowered mutants with higher amounts of aromatic compounds (methyl benzoate), suggesting that specific floral color-scent associations could be a consequence of conserved biochemical pathways (Armbruster, 2002), and these associations may not be dissociated, or formed, by natural or artificial selection alone (Majetic et al., 2007).

In this study, we investigated color-scent associations in the three forms of the floral polymorphic *Plumeria rubra* L. (Apocynaceae) originating from Central America, showing three different distinct color morphs. We analyzed the scent production of the three color forms of *P. rubra* L. and attempted to determine whether the floral color and scent were associated in this species. We tried to address three questions:

(1) What is the scent composition difference among the three forms of *P. rubra* L. flowers?
(2) Are there specific color-scent associations among the distinctive color forms?
(3) Does floral scent vary significantly among individuals within the color form of *P. rubra* L.?

2. Materials and methods

2.1. Study species and sites

The study was carried out from June to July 2015 in the full-blossom period of three *P. rubra* L. forms from a cultivated population in a garden of the University of Honghe, Yunnan Province, China (23°21′23″N, 103°25′28″ E; asl. 1315 m). These plants produced three obvious floral colors, which are white, yellow and red (Fig. 1). The form *P. rubra* f. *acutifolia* has white flowers with yellow centers, *P. rubra* f. *lutea* has yellow flowers fading to white at the edge as they mature and *P. rubra* f. *rubra* has pink flowers of varying intensity with a tangerine-yellow center.

2.2. Volatile collection and analysis of floral scents

Floral scents emitted from the three forms were collected using the dynamic head space adsorption method with a preset flow velocity of 350 ml/min during the sunniest time of the day between 09:00 and 12:00 (Beijing time, UTC+8). The collection was performed in the field, and intact flowers were carefully enclosed in a modified vacuum dryer. The flower peduncles were covered with absorbent cotton soaked in a 10% sucrose solution. The scent-containing air was drawn through glass cartridges containing adsorbent Porapak Q (150 mg, mesh 60/80; Waters Associates, Milford, MA, USA) for 2–3 h during daytime using a pump with an outlet flow rate of 350 ml/min. The duration of trapping activities depended on the relative strength of the scent to the common feelings of the human nose. The ambient air pumped into the vacuum dryer was purified sufficiently with advanced activated carbon. To identify the background contamination of the chemical samples, ambient air (purified air) was collected as a control. Due to marginal differences in flower size, ten flowers of each plant were collected randomly and combined into one sample. Five to seven individual plants of each form were used to obtain enough representative samples. Cartridges were conditioned before sampling by washing them with 400 ml dichloromethane, n-hexane and acetone for 4 h. The adsorbed scent components were eluted with ca. 0.8 ml dichloromethane and were collected in a 1.5 ml Agilent vial (Agilent Corporation, USA). Then, the vials were stored in a −80 °C freezer until the final analysis was conducted.

The extracts were analyzed using coupled gas chromatography and mass spectrometry (GC-MS). In total, 17 samples were analyzed using an Agilent HP 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with an HP-5MS column (30 m × 0.25 mm, 0.25 μm film thickness), linked to an HP5973 mass spectrometer. Helium (He) was used as a carrier gas at a flow of 1 ml/min, and the injector temperature was set to 250 °C. The column temperature was first set at 40 °C and was then programmed to 250 °C at a rate of 3 °C/min. Compounds were identified by comparing their retention times (RT) and mass spectra with those of authentic compounds or with MS spectra from the Wiley 7n.1 mass spectral library and the associated retention indices reported both in the NIST Chemistry Web Book (http://webbook.nist.gov) and in the RI database 42 (Adams, 2001).

Kovats retention indices were calculated using the formula:

$$I_x = 100_n + 100 \times (t_x\text{-}t_n)/(t_{n+1}\text{-}t_n)$$

Where I_x is the retention index of the compound of interest, t_x is the retention time of the compound of interest, t_n and t_{n+1} are the retention times of heading and trailing n-alkanes, respectively, (Sigma-Aldrich Co., St. Louis, MI, USA), and n is the number of carbon atoms of heading n-alkane peak (Chen et al., 2012; da Cunha et al., 2013; van Den Dool and Dec Kratz, 1963).

The proportional abundance of the compounds within the floral scents, which was defined as the relative amount with respect to aggregate peak area but excluding contaminants, was calculated based on the absolute amounts of the compounds.

2.3. Floral color measurements

The flower colors of three forms were evaluated by comparison with the Royal Horticultural Society color chart (n = 12). In addition, we measured the floral color of three forms from 300 to 700 nm by a spectrophotometer (USB2000+; Ocean Optics, Dunedin, FL, USA) equipped with a Xenon Pulse X2 lamp light source, following the methods of Chittka and Kevan (2005). The areas of the petal and nectar guides of eight red flowers from eight plants, 21 white flowers from four plants and 20 yellow flowers from five plants were measured separately. Spectral readings were replicated three times for each sample and were then averaged before further analysis. Spectral curves of the petal and nectar guides for these three morphs are presented. The spectrometric data were then analyzed using the method of AVICOL v.6 (Gomez, 2006).

2.4. Statistical analysis

To characterize the floral scent dissimilarity with distinct floral color, we carried out a nonmetric multidimensional scaling (NMDS)

(a) *P. rubra* f. *acutifolia* (b) *P. rubra* f. *lutea* (c) *P. rubra* f. *rubra*

Fig. 1. Three natural floral colors existing in *Plumeria rubra* L: a, white; b, yellow; c, red flower morphs.

ordination based on a matrix of Bray-Curtis dissimilarities calculated on the relative proportions of odor compounds (in percentage of the total blend). The stress value generated by the NMDS analysis reflects how well the ordination summarizes the observed distances among the individuals. The dissimilarity indices of floral scents were also quantified through one-way SIMPER analysis in PAST (Hammer et al., 2001). Additionally, cluster analyses through stratigraphically constrained clustering were performed to identify quantitatively similar chemical groups of the all samples among the different individuals and forms using the same Bray-Curtis index of similarity. Among the algorithms for hierarchical clustering, we selected the unweighted pair group method using averages (UPGMA), which is a method conventionally used in ecology (James and McCulloch, 1990; Wolda, 1981). One-way ANOSIM (analysis of similarities) global tests were then applied to test for significant differences between subgroups produced by the cluster analysis. The chemical components data of the three forms were log normalized through $Log_{10}(X+1)$ transformation to bring out category features of the original percentage data and to reduce the weight of those of a few very dominant components. We also used a paired *t*-test to test whether the Bray-Curtis similarity distance indices of the chemical components among the three forms produced by the ANOSIM data were significantly different from each other.

To determine the chemical compounds of the floral scents that are statistically associated with certain forms, an indicator compound analysis (ICA) with 999 random permutations was performed. The computed indicator value of each compound reflects both its relative abundance and its relative frequency. The associated *P*-values determined whether specific compounds are significant indicators of certain forms. (Cáceres and Legendre, 2009). Finally, the contribution of each compound with an ICA that was statistically significant among individuals was determined using PCA based on the relative amount of each chemical compound through $Log_{10}(X+1)$ transformation in PAST. A variance-covariance matrix of the floral scents based on relative amount was used, and the obtained Jolliffe cut-off value provided an informal indication of how many components should be considered significant (Jolliffe, 1986). The coefficient of each principal component implies the contributions of each compound within a form. The absolute values of coefficients showed their relative contributions.

3. Results

3.1. The color differences of petals

Our field investigation revealed that the petal spectrum of five individuals of the red *P. rubra* L. showed significant differences relative to the white and yellow forms. Corolla color ranged from red (72C) to yellow (4C) and white (155D) (Fig. 2). Based on the spectral curves, the petal color of the white and yellow forms showed similar parameters, and both of these colors had a much higher reflectance than the petal part of the red form in the wavelength range of 400–600 nm. Both color morphs of white and yellow forms show similar spectral curves at the center nectar guide part of the flower, indicating a similar color of foraging guides in the eyes of pollinators. The nectar guide of the red form had a much lower reflectance than the petal part, with ranges of 400–480 nm and 600–700 nm, respectively, and the reflectance of the red nectar guide was also significantly lower than that of the white and yellow forms (Fig. 2).

3.2. The chemical composition differences of floral scents

The NMDS ordination was characterized by a low stress value (stress = 0.1392), which illustrated the inter-morph floral scent component differentiation with distinct floral color (Fig. 3a). The results of one-way SIMPER further supported and quantified the inter-morph dissimilarity of floral scents, which reached 35.73%, and two different scent compounds contributed greatly, namely, lilac alcohol A (28.62%) and lilac aldehyde A (28.15%). However, the intra-morph dissimilarity of floral scent from different individuals was lower, at 14.09% for yellow morph individuals and 16.73% for red morph individuals. More details are presented in Table S1 within the Supplementary Information file. The overlap suggests that *P. rubra* L. with red petals has relatively higher composition similarity indices than the yellow and white forms. The analysis of intraspecific levels of variance dispersion with Bray-Curtis distances among floral scent samples showed that there were significant differences within the three forms (ANOVA $F_{2,38} = 3.959$, $P = 0.02743$), indicating that the white form had the highest intraspecific dissimilarity, the yellow form ranked second, and the red form had the lowest intraspecific dissimilarity index (Fig. 3b). Significant inter-specific differences in the variance dispersion of chemical contents for floral scents are shown in Fig. 3b.

The cluster analysis dendrogram based on the quantitative similarities of floral scent components measured through relative percentage is shown in Fig. 4a. As supported by global one-way ANOSIM tests, meaningful differences between groups occurred at an almost similarity of 0.53 and generated 2 subgroups (global $R = 0.695$; $P < 0.001$). Subgroup 1 was represented by five individuals of the red form. Subgroup 2 included the five individuals of the white form and seven individuals of the yellow form. Subgroup 2, which was clustered by white and yellow form individuals together, could also be further divided into two subgroups clearly defined by color differences. The global one-way ANOSIM tests showed that the cluster of the five red individuals had significant differences with both the white and yellow *P. rubra* L. forms ($P < 0.01$).

In this study, ICA analysis showed that the association between the scent and color in the red form was characterized by five fatty acids, including one diacetone alcohol, two butanols and two methylbutanals. However, the floral scent differences between the white and yellow morphs were mainly explained by benzenoid compounds. In the white

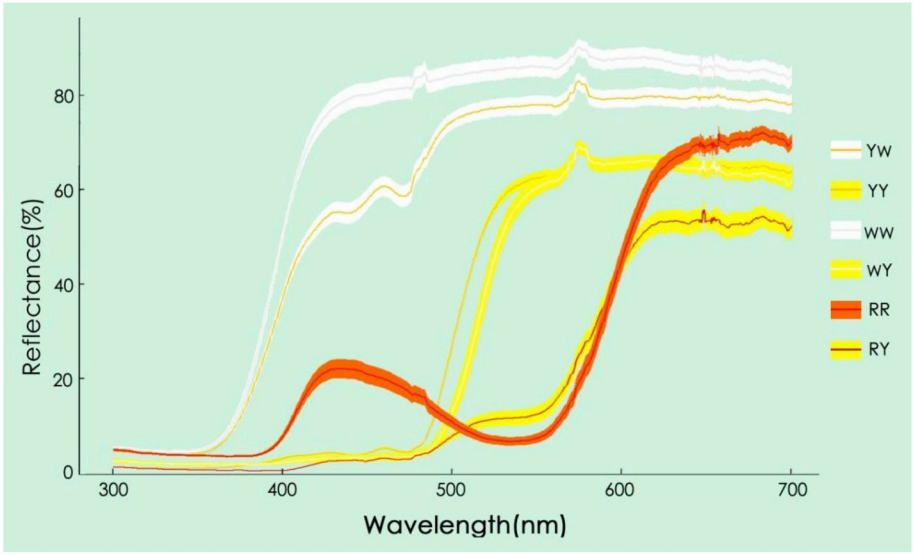

Fig. 2. Flower reflectance spectra of different color morphs among three different *Plumeria rubra* L. forms. (YW, white petal of yellow form *P. rubra* f. *lutea*; YY, yellow nectar guide of yellow form; WW, white petal of white form *P. rubra* f. *acutifolia*; WY, yellow nectar guide of white form; RR, red petal of red form *P. rubra* f. *rubra*; RY, yellow nectar guide of red form).

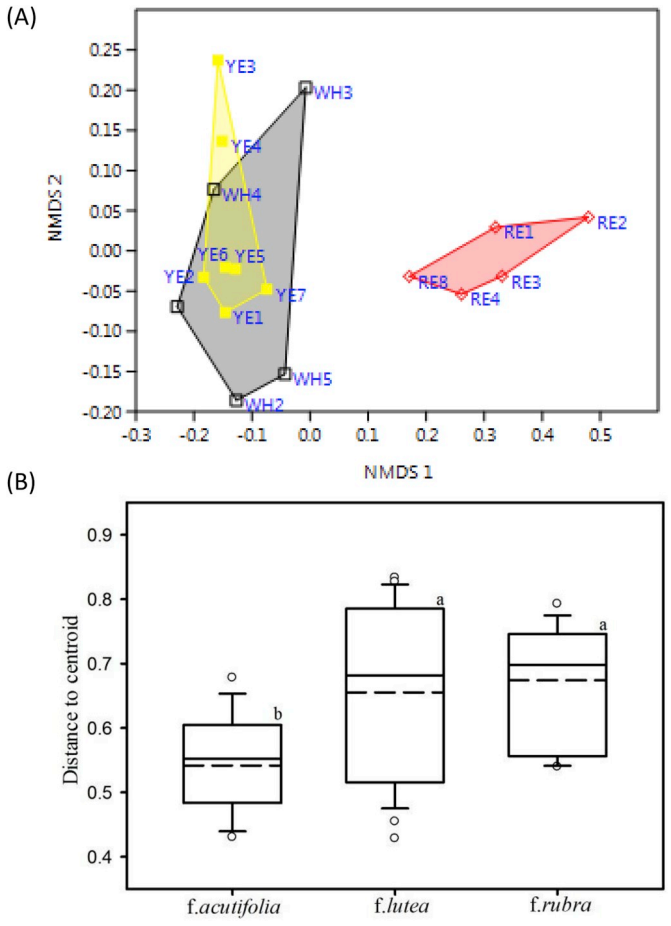

Fig. 3. Floral scent differentiation among the three *Plumeria rubra* L. varieties investigated. A: nMDS biplot of floral scent differentiation based on a matrix of Bray–Curtis dissimilarities calculated on the relative proportions of odor compounds (in % of total blend). B: Boxplot of intraspecific levels of floral scent dispersion using Bray–Curtis distances among samples (different letters on top of boxplots indicate significant differences with a = 0.05, tested with Tukey HSD).

form, benzenemethanol and phenylmethanal were obviously higher than in the yellow form. However, the relative amount of methyl benzoate in the white form was significantly lower than in the yellow form (Table S1; Fig. 2). The red form had much higher geraniol and dl-Limonene contents than either the white or yellow forms ($P < 0.05$). However, there was no significant difference in the levels of these compounds in the white and yellow forms ($P > 0.05$). Additionally, both the yellow and white forms emitted significantly higher linalool than the red form ($P < 0.01$).

Principal component analysis was carried out using all the identified compounds among the three *P. rubra* L. forms. Two principal components were found, which explained 44.56% and 13.45% of the total variances (Fig. 4a and b). The scent profiles of the three *P. rubra* L. forms were dominated by distinct floral scent types: alcohol and butanol for the red flower morph and methyl benzoate for the white and yellow morphs. Fatty acid derivatives, benzodiazepines and terpenoids contributed greatly to the inter-morph differentiation of the three morphs (Fig. 4a and b).

4. Discussion

The results showed that three different color morphs of *P. rubra* L. forms produced two significantly different kinds of odors, and the odors emitted by the white individuals corresponded to the yellow form. The red flower morph emitted significantly distinct aromatics with diverse fatty acid derivatives compared to the white and yellow forms. The smell and optesthesia of these forms are contradictory during the night, especially for those nocturnal hawkmoths distinguishing red flowers from white and yellow through aromatics differences. Floral polymorphisms are complex and strongly affected not only by pollinators but also by other selective agents, such as herbivores, abiotic factors or even by pleiotropic effects of both natural and artificial selection acting on other traits (Dormont et al., 2010; Mark D Rausher, 2008; Schaefer and Ruxton, 2009; Schaefer et al., 2004). In typical deceptive pollination plants, it seems there are greater selection pressures considering their dependence on pollination agents from the surrounding fluctuant environments. However, cultivated *P. rubra* forms were probably an exception to this condition due to the heavy impacts from human and artificial breeding.

The flowers of wild *P. rubra* L. are hermaphroditic with a consistently low natural fruit set (less than 0.1%). Bawa (1974) found that self-incompatible. *P. rubra* L. plants usually do not set fruit without the help of animal pollinators in native Central America (Haber, 1984; Tremblay et al., 2005). Introduced and cultivated *P. rubra* L. forms are no exception to this rule, and they rarely show fruit settings. Most of the forms were cultivated and reproduced by the cutting reproduction method, and there is no great potential advantage for pollinator agents

to select specific floral trait combinations that are attractive for them during the night. Johnson et al. (2003) found that the neighboring rewarding floral diversity could shape pollinator preferences and lead to mimicry for nectarless species. For example, var. lilacina of *Calanthe sylvatica* (Orchidaceae, Calanthe) could take advantage of the

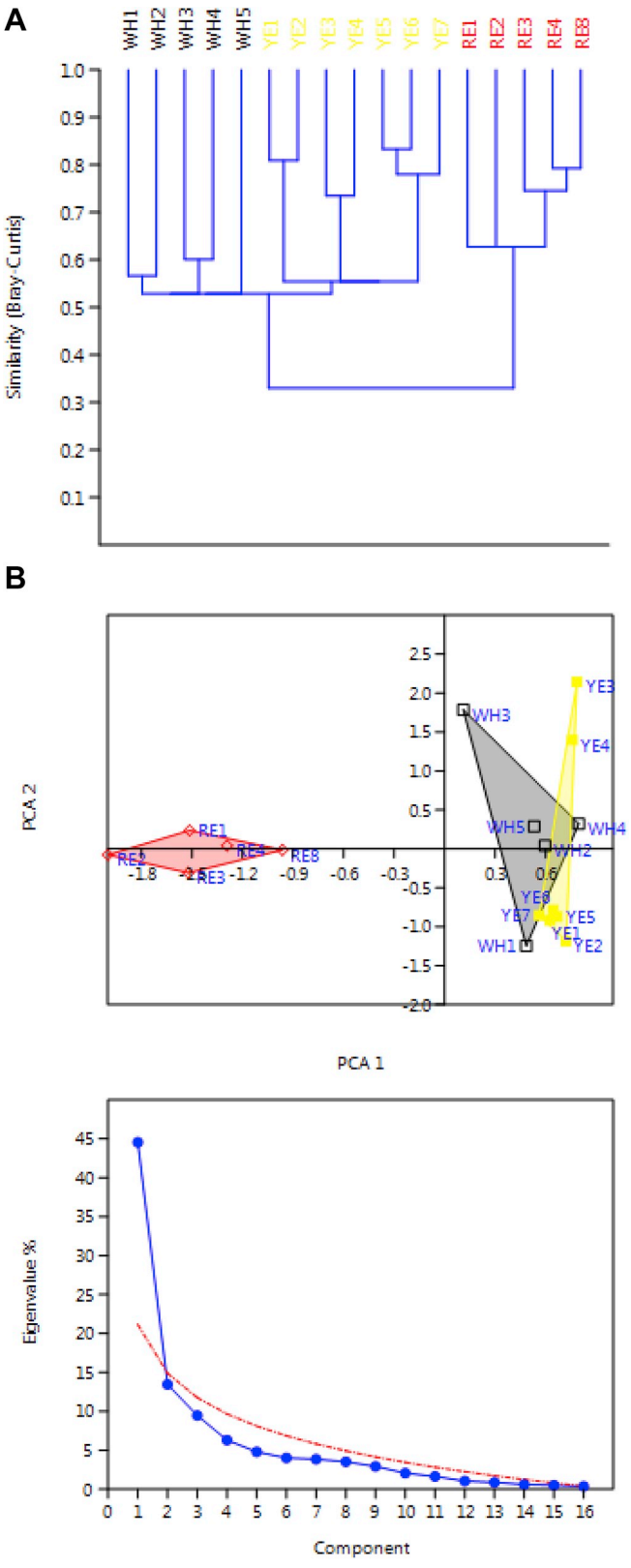

Fig. 4. (A) Quantitative similarity cluster analysis of floral components from the three *Plumeria rubra* L. varieties investigated at the sampling locations (sample codes WH, YE and RE represent the white, yellow and red forms of Plumeria rubra L. respectively), generated from the Bray-Curtis index using the unweighted pair group method with arithmetic mean (UPGMA). The dendrogram shows two subgroups at similarity levels of > 0.5. **(B)** Principle Component Analysis (PCA) of floral components from the three P *rubra* L. varieties investigated at the sampling localities: () *P. rubra* L.; () *P. rubra* L. Acutifolia; () *P. rubra* L. Lutea. Cumulative variation in the original dataset explained by ordination is 58.01% (Axis 1 = 44.56%, Axis 2 = 13.45%).

pollinators attracted by the rewards from the surrounding shrub *Chassalia coralioides*, which often blooms in the same population and presents flowers of the same color (Juillet et al., 2010). Therefore, the color-scent associations documented here could also be independent of each other. This relationship might be resulting from divergent environmental conditions in different regions of introduction or from selective pressures on each trait exerted by forces unrelated to pollinators.

Many studies have attributed complex and diverse flower morphology, especially flower shape, to adaptation for various pollination body shapes (Johnson and Steiner, 1997; Juillet et al., 2007, 2010). Compared to typical deceptive pollination plant orchid species, the three *P. rubra* L. forms with large infundibula form flowers and tiny inflorescence bracts did not evolve such highly diverse flower morphologies, which were regarded as adaptations to the various insect body shapes for reaching deep corolla tubes and getting pollen grains. However, different *P. rubra* L. forms might follow the strategy of 'diverse and complex combination of petal color and odors' to attract accidental insect species from the surrounding environment with many other rewarding flowering species. Our results suggested that the role of color and scent associations might be much more important for *P. rubra* forms. The red form, which was not obviously used for insect pollination, emitted more aromatics with alcohol. However, the white and yellow forms emitted more components of benzoate and terpenes, respectively. The proportional assemblages of benzoate and terpenes also reflected the variation of color morphology of white and yellow forms.

The red form of *P. rubra* L. emitted much more fatty acid derivatives (especially 2-methyl-1-butanol and 2-methylbutanal), which play major roles in the formation of sweet and aromatic taste to attract general insect pollinators. Methyl benzoate was the major component of the white and yellow forms. The results showed that the relationships between color and scent on most of the flowering plants are partly inherent because the two kinds of traits rely on shared biosynthetic pathways (Armbruster, 2002; Majetic et al., 2007), implying that any mutation in a gene coding for an enzyme or a regulatory element of these pathways could have an impact on both color and scent. Knudsen et al. (2006) found that volatile organic compounds involved in floral scent are dominated by fatty-acid derivatives, (mono or sesqui) terpenoids and phenylpropanoids/benzenoids. Wu (2008) confirmed that terpenoids (carotenoids, mono and sesquiterpenoids) are all synthesized from the same precursor isopentenyl pyrophosphate and dimethylallyl pyrophosphate. Dudareva et al. (2004) and Zvi et al. (2008) also confirmed that phenylpropanoid volatile compounds and flavonoid pigments both originate from the same phenylpropanoid biosynthetic pathway. In fact, in both terpenoids and phenylpropanoids, volatiles are early products of the pathway, while pigments are produced at later steps. The fact that biosynthetic pathways are shared suggests the potential choice for correlated responses in color and scent to a single change in biochemistry. For instance, the removal of petal pigment by antisense suppression of the flavanone 3-ydroxylase gene in the anthocyanin biosynthetic pathway led to higher emission of an aromatic volatile in *Dianthus caryophyllus* (Zuker et al., 2002). The relationships between red color and high emission of more fatty acid derivatives and between white and yellow color and high emission of linalooland methyl benzoate, as found in the present study, confirm that specific innate associations exist in cultivated *P. rubra* L. forms.

Our results showed that fatty derivatives are the major components of the red form of *P. rubra*, while benzoate and terpenes are the major components of the white and yellow forms. This difference is a good indication for color morphology among different *P. rubra* L. forms. The present study supports the hypothesis that intrinsic relationships exist for flower color morphs and scent chemical components. Various mechanisms responsible for this pattern have been suggested, but which of these are prevalent under which conditions remains an open question. *P. rubra* L. plants are important landscape ornamentals and fragrances around the tropical and subtropical world. Our results on the natural floral scents of the three *P. rubra* L. forms might provide information for understanding floral scent and color associations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bse.2019.05.005.

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