Postprint: Pollen Morphology and Classification of 22 Hibiscus Cultivars

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Abstract

To investigate the diversity of pollen morphology and genetic relationships among different cultivars of *Hibiscus syriacus*, this study examined pollen from 22 cultivars using scanning electron microscopy to observe morphological characteristics and exine ornamentation, followed by R-type cluster analysis and principal component analysis to extract appropriate indices for UPGMA cluster analysis. The results were as follows: (1) The pollen grains of *Hibiscus syriacus* were all single, subspherical, with diameters of 148.98 m-111.65 m; the surface exhibited spinulate ornamentation, with spine lengths of 27.42 m-14.79 m, the spine apices were relatively sharp, and scattered pores were uniformly distributed around the fine spines; the pollen surface was covered with granular protrusions, and the germination pores had irregular shapes. (2) Following principal component extraction from the measured indices, UPGMA cluster analysis was performed. When the Euclidean average distance threshold was 6, the 22 *Hibiscus syriacus* cultivars were divided into six major groups: single-petaled blue-purple cultivars such as 'Blue Bird', 'Single Purple Pink', and 'Xiye'showed relatively close genetic relationships; semi-double cultivars such as 'Lavender Chiffon', 'China Chiffon', and 'Pink Chiffon' showed relatively close genetic relationships; while pink-white single-petaled cultivars such as 'Muqiao' and 'Hanbo' showed relatively close genetic relationships. This study suggests that within *Hibiscus syriacus* species, blue-purple cultivars are relatively more primitive, white semi-double cultivars are secondary, purple semi-double cultivars are relatively more evolved, and white single-petaled cultivars exhibit the highest degree of evolution. These results provide a basis for identifying intraspecific genetic evolution, taxonomic status, and genetic relationships in *Hibiscus syriacus*.

Full Text

Pollen Morphology and Numerical Taxonomy of 22 Hibiscus syriacus Cultivars

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Abstract

To investigate pollen morphological diversity and genetic relationships among different Hibiscus syriacus cultivars, this study examined pollen grains from 22 cultivars using scanning electron microscopy to observe morphological characteristics and exine ornamentation. R-type cluster analysis and principal component analysis were subsequently employed to extract appropriate indicators for UPGMA cluster analysis. The results were as follows: (1) All H. syriacus pollen grains were single and nearly spherical, with diameters ranging from 148.98 m to 111.65 m. The surface exhibited spinulose ornamentation measuring 27.42 m to 14.79 m in length, with sharp spine tips and scattered pores uniformly distributed around the fine spines. The pollen surface was covered with granular protrusions, and aperture shapes were irregular. (2) Following principal component extraction from measured indicators, UPGMA cluster analysis divided the 22 H. syriacus cultivars into six major groups at a Euclidean average distance threshold of 6. Single-petal blue-violet cultivars such as 'Bluebird' , 'Dan Ban Zi Fen' , and 'Xi Ye' showed close genetic relationships; semi-double cultivars including 'Lavender Chiffon' , 'China Chiffon' , and 'Pink Chiffon' were closely related; while pink-white single-petal cultivars like 'Wood Bridge' and 'Hamabo' formed another closely related group. This study suggests that within H. syriacus, blue-violet cultivars represent relatively primitive forms, pink-white semi-double cultivars are intermediate, purple semi-double cultivars are relatively evolved, and white single-petal cultivars are the most highly evolved. These findings provide a basis for identifying genetic evolution, taxonomic status, and phylogenetic relationships within H. syriacus.

Keywords: *Hibiscus syriacus*, scanning electron microscope, pollen morphology, principal component analysis, cluster analysis

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Introduction

Hibiscus syriacus is a commonly used shrub or small tree in Chinese landscaping. The species exhibits diverse flower colors including pure white, cream, purple, pink, and red, with three petal types: single, semi-double, and double. Its exceptionally long flowering period from June to October fills the gap of flowering woody plants in northern Chinese gardens during summer and autumn

(Liu et al., 2008). Although over 200 cultivars have been developed in Europe, America, and Korea, with nearly 40 commonly used in Western horticulture, systematic research on *H. syriacus* classification and breeding remains limited in China. Continuous artificial hybridization and natural selection have resulted in confused cultivar relationships, unclear origins, ambiguous genetic affinities, and uncertain classification and evolutionary relationships. Therefore, studying cultivar classification and genetic relationships is crucial for facilitating inter-cultivar hybridization and new cultivar development.

Pollen morphology varies considerably among different plants and can reflect genetic differences. Under genetic control, pollen morphology exhibits conservative, reliable, and stable characteristics (Li et al., 2018). Previous palynological studies on Malvaceae plants include EL Naggar S M (2010) investigating pollen morphology of 21 species across 10 genera, and BiBi N (2010) examining nine species across six genera, both concluding that pollen morphological traits and exine ornamentation patterns provide valuable references for species delimitation within the family. Recent studies on other genera have also revealed intraspecific pollen morphological differences that serve as important criteria for cultivar classification and phylogenetic research (Zhang et al., 2018; Liu et al., 2018; Luo et al., 2017). Consequently, pollen morphological observation and palynological marker studies are significant for investigating genetic diversity and phylogenetic relationships among plant cultivars.

To elucidate genetic relationships among *H. syriacus* cultivars, this study examined pollen from 22 cultivars using scanning electron microscopy to observe morphological variations. Cluster analysis based on measurement results was conducted to explore intraspecific phylogenetic relationships, aiming to provide palynological theoretical support for *H. syriacus* cultivar classification and genetic relationship studies.

Materials and Methods

1.1 Materials

Mature pollen from 22 *H. syriacus* cultivars was selected as experimental material. Cultivar names and sources are detailed in Table 1.

Table 1 Names and sources of 22 *Hibiscus syriacus* cultivars used in this study

1.2 Methods

1.2.1 Pollen Collection and Storage Healthy, pest-free *H. syriacus* samples were selected, and fresh pollen was collected and immersed in 4% glutaraldehyde solution, then stored at 4°C pending examination (Erdtman G, 1978). Pollen was collected from three different individual plants per cultivar, with three replicates.

1.2.2 Dehydration Pretreatment Prior to SEM observation, stored pollen samples were removed and rinsed three times with deionized water for 6, 7, and 8 minutes respectively, followed by sequential dehydration in 50% ethanol for 14 minutes → 70% ethanol for 14 minutes → 85% ethanol for 14 minutes → 95% ethanol for 15 minutes → 100% ethanol for 15 minutes.

1.2.3 Drying Treatment The CO critical point dryer was activated and cooled to 10°C. Samples were placed inside, liquid CO was introduced, and after 27 minutes the CO liquid was released. This procedure was repeated three times. Liquid CO was then reintroduced, heated for 45 minutes, the main valve closed, and samples were removed after gas had been slowly released.

1.2.4 SEM Observation Pollen samples were sputter-coated with gold and observed using a HITACHI SU8010 scanning electron microscope. Representative pollen grains were photographed at 200× magnification for population views, 500× for individual grains, and 3000× for detailed surface ornamentation.

1.2.5 Data Analysis Following the methods of Peng (2018) and Liu et al. (1987), various pollen morphological indicators were measured including pollen diameter, spine length, and inter-spine distance. SPSS 22.0 was used for significance testing of measured indicators according to Tukey' s test. Variation range was expressed as minimum and maximum values. Coefficient of variation (CV%) = (S × 100%)/$\bar{x}$, where S represents standard deviation. R-type cluster analysis was performed on selected indicators based on calculated means, principal components were extracted, and UPGMA cluster analysis was conducted using the obtained principal components as factors.

Results

2.1 Basic Pollen Characteristics

Based on measurement indicators used by Peng (2018) and Liu et al. (1987) for Malvaceae pollen morphology classification, SEM results of 22 *H. syriacus* pollen samples were analyzed. As shown in Plate I, pollen from all 22 cultivars was single and nearly spherical, with spinulose ornamentation distributed across the surface. Spines were conical with blunt tips, and scattered pores were uniformly distributed around fine spines. The pollen surface featured granular protrusions, aperture shapes were irregular, and apertures were exposed on the pollen surface after fine spine detachment.

2.2 Pollen Size

Overall, *H. syriacus* pollen existed as single, nearly spherical grains with a mean diameter of 122.54 m and a coefficient of variation of 1.01%. 'Purple French

Carbet' exhibited the largest diameter at 148.98 m, while 'Bluebird' showed the smallest at 111.65 m.

All 22 cultivars displayed spinulose ornamentation on the pollen surface, with varying spine thickness and distribution patterns. Mean spine length was 21.18 m (CV = 1.56%), with 'Dan Ban Zi Fen' showing the longest spines at 27.42 m and 'Blue Chiffon' the shortest at 14.79 m. Mean spine base width across 22 cultivars was 10.44 m (CV = 1.79%), with 'Mint' showing the maximum base width of 13.26 m and 'Purple Pillar' the minimum at 6.84 m. Mean inter-spine distance was 30.21 m (CV = 1.14%), with the maximum distance of 36.18 m observed in *H. syriacus* and the minimum of 24.09 m in 'Blue Chiffon' . Regarding the spine length/base width ratio, 'Purple Pillar' showed the highest value at 2.81, indicating the most slender spines, while 'Mint' showed the lowest ratio at 1.42, indicating the stubbiest spines. 'Bluebird' exhibited the largest pollen diameter/spine length ratio, reflecting relatively short spines, whereas 'Dan Ban Zi Fen' showed the smallest ratio, reflecting relatively long spines. This latter indicator displayed the highest coefficient of variation at 2.24%.

Table 2 Pollen grain size and surface ornamentation characteristics of 22 *Hibiscus syriacus* cultivars

2.3 R-Type Cluster Analysis and Principal Component Analysis of Six Pollen Morphological Indicators

R-type cluster analysis assesses correlations among selected indicators to identify representative traits for subsequent analysis. The R-type cluster analysis results shown in Figure 1 [Figure 1: see original paper] indicate that the morphological indicators are relatively dispersed, suggesting the selected indicators are appropriate for further Q-type cluster analysis. Principal component analysis of six *H. syriacus* pollen exine morphological indicators revealed that these could be divided into two principal components. The first principal component included spine length, pollen diameter, spine length/spine base width, and pollen diameter/spine length, with pollen diameter/spine length and spine length/spine base width contributing most significantly. The second principal component included spine base width and inter-spine distance. The cumulative contribution rate of these six indicators reached 77.611%, indicating they could comprehensively reflect *H. syriacus* pollen exine morphological information. Combined with R-type cluster analysis results, the six selected morphological indicators were representative and suitable for Q-type cluster analysis of *H. syriacus* pollen.

2.4 Q-Type Cluster Analysis of Pollen Exine Morphology in 22 *Hibiscus syriacus* Cultivars

Q-type cluster analysis based on these six indicators classified the 22 pollen types. At a Euclidean average distance threshold of 6, the cultivars were divided into six major groups. Group I contained only 'Blue Chiffon' , and Group II

contained only 'Mint', both characterized by relatively wide spine bases and large pollen diameter and pollen diameter/spine length values. Group III comprised 'Bluebird' alone, while Group IV included only 'Dan Ban Zi Fen', which featured short spines, wide spine bases, long inter-spine distances, large pollen diameter, small spine length/base width values, and large pollen diameter/spine length values. Group V included four single-petal pink-purple cultivars: 'Purple Pillar', 'Xi Ye', 'Pink Giant', and 'Yan Zhi Hong', all showing relatively large spine length/base width values. Group VI exhibited relatively small spine length/base width values and large pollen diameter/spine length values, primarily including pink-white single-petal cultivars such as 'Red Heart', 'Hamabo', and 'Wood Bridge', three purple semi-double cultivars ('Purple French Carbet', 'Rou Fen Hong Xin', and 'Lavender Chiffon'), and two pink-white semi-double cultivars ('Pink Chiffon' and 'China Chiffon').

Figure 1 Dendrogram of R-type cluster analysis

[Figure 1: see original paper]

Table 3 Eigenvalues and contribution rates of test indexes for *Hibiscus syriacus*

Table 4 Normalized characteristic vectors from principal component analysis

Figure 2 Pollen morphology dendrogram of 22 *Hibiscus syriacus* cultivars

[Figure 2: see original paper]

Note: Numbers 1-22 on the ordinate represent the 22 H. syriacus* cultivars, detailed in Table 1.*

Discussion

3.1 Systematic Evolutionary Characteristics of *Hibiscus syriacus* Pollen

Smaller pollen volume indicates less nutrient competition with the plant and higher evolutionary advancement (Yang et al., 2014; Zhou et al., 2005). The 22 *H. syriacus* cultivars exhibited large pollen grains ranging from 148.98 m to 111.65 m. Exine ornamentation features can reflect plant evolutionary patterns; generally, smooth pollen surfaces are more primitive, followed by granular protrusions, then rod-shaped and spinulose patterns, finally evolving into reticulate, rugulate, and striate patterns. The evolutionary trend proceeds from large, rough-surfaced pollen to small, smooth-surfaced pollen. Overall, *H. syriacus* pollen is large, nearly spherical, with a single irregular aperture and spinulose ornamentation of varying thickness and length, indicating a relatively primitive plant group.

3.2 Evolutionary Position of *Hibiscus syriacus* Within Malvaceae

Pollen morphology is genetically controlled and stable, being less affected by environmental factors, making palynological markers valuable for plant clas-

sification and phylogenetic analysis. At higher taxonomic levels (family and subfamily), pollen morphology shows greater differences, while differences are smaller among genera within the same family and minimal among cultivars with more similarities. Previous research indicates that within Malvaceae, exine ornamentation evolution trends include increases in size, transitions from tricolpate to polyporate apertures, and changes from basally swollen short spines to basally flat long spines (Sun et al., 1993; EL NAGGAR S M, 2004; EI NAUUAR S M & SAWADY N, 2008; Peng et al., 2018). The observed *H. syriacus* cultivars exhibited large pollen grains with irregular scattered pores and long, slender spines with flat bases, consistent with relatively evolved exine ornamentation characteristics within Malvaceae. Therefore, *H. syriacus* represents a relatively evolved group within the family.

3.3 Taxonomic Significance of *Hibiscus syriacus* Pollen Morphology

Using six pollen exine morphological indicators derived from R-type cluster analysis and principal component analysis, Q-type cluster analysis was performed on 22 *H. syriacus* cultivars as a variable classification method. Results revealed that pollen cluster analysis correlated with certain floral morphological traits. In Group V, single-petal blue-violet cultivars 'Bluebird' , 'Dan Ban Zi Fen' , 'Xi Ye' , and 'Pink Giant' clustered together, indicating that pollen morphological features correlate with flower color and type. Xiao et al. (2019) conducted comprehensive analyses of 27 *H. syriacus* cultivars using morphological, palynological, and ISSR molecular markers, finding consistency among these approaches, which aligns with our results. Zhang and Shi (2009) studied evolutionary relationships among five *H. syriacus* cultivars from a wood anatomy perspective, concluding that purple-flowered single-petal *H. syriacus* represents the most primitive group, which subsequently differentiated into tree peony *H. syriacus* and then purple-flowered double-petal *H. syriacus*. In our study, at a Euclidean average distance threshold of 3, *H. syriacus* clustered with semi-double cultivars 'Lavender Chiffon' , 'China Chiffon' , and 'Rou Fen Hong Xin' , which show high morphological similarity to tree peony *H. syriacus* as pink semi-double cultivars, suggesting these three cultivars represent relatively primitive forms initially differentiated from purple single-petal *H. syriacus*. Pink-white single-petal cultivars such as 'Wood Bridge' , 'Hamabo' , and 'Red Heart' showed close genetic relationships among themselves but relatively distant relationships with *H. syriacus*, indicating this flower type is relatively evolved. Consequently, we propose that within *H. syriacus*, blue-violet cultivars are relatively primitive, pink-white semi-double cultivars are intermediate, purple semi-double cultivars are relatively evolved, and white single-petal cultivars, being genetically distant from *H. syriacus*, represent the highest evolutionary level. Pollen morphology correlates with flower morphology, though the latter may be influenced by environmental factors; both can serve as criteria for systematic classification of *H. syriacus*.

In summary, *H. syriacus* belongs to a relatively primitive group overall, but

represents a relatively evolved group within Malvaceae. Its pollen morphology correlates with flower color and type. Exine ornamentation is genetically controlled and relatively stable (Zhao, 2014); however, relying solely on palynology for determining inter-cultivar relationships and classification has limitations and lacks comprehensiveness. Future research should integrate morphological markers, molecular markers, and other approaches for comprehensive analysis.

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Note: Figure translations are in progress. See original paper for figures.