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Article

Optimum Discrimination on the Subsection Taxonomy of Wild Tree Peony Species in China Using Pollen Characteristics

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Abstract: Pollen characteristics have some significance for plant taxonomy classification. We intend to explore a more concise index value for the subsection taxonomy with the pollen morphology and pollen viability determined from all nine wild tree peony species, including 18 populations native to China. We observed the pollen morphologic characters by scanning electron microscopy and measured the pollen viability in vitro. The results showed that the pollen polar length is the decisive characteristic in distinguishing the two subsections in section *Moutan* belonging to Paeoniaceae. The pollen polar length of five species belonging to subsect. *Vaginatae* is longer than 43 µm, while that of the four species belonging to subsect. *Delavayanae* is shorter than 43 µm. Meanwhile, the differences in pollen viability between the two subsections also play an auxiliary role in the classification of tree peony. The germination rate of the populations in subsect. *Vaginatae* is mostly greater than those of the populations in subsect. *Delavayanae*. The pollen germination rate of populations in subsect. *Vaginatae* is commonly more than 50%, while that of populations in subsect. *Delavayanae* less than 50%. Our study established taxonomic evidence between subsections in section *Moutan* and compared the pollen viability of subsect. *Vaginatae* and subsect. *Delavayanae*.

Keywords: Paeoniaceae; classification; section *Moutan*; pollen morphology; pollen viability

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1. Introduction

Tree peony, belonging to section *Moutan* DC. of the genus *Paeonia* L. (Paeoniaceae), is a perennial woody shrub native to China that has been considered a traditional ornamental and medicinal plant since the Han Dynasty (202 BCE–220 CE) [1]. Marked by a variety of species and colors, the tree peony is considered a symbol of prosperity and fortune, which makes it one of the candidates for the national flower of China. All wild tree peonies are endemic to China, but *Paeonia suffruticosa* is commonly cultivated throughout the north temperate region. However, the taxonomy of the group had been neglected before the 1990s. Since 1990, a number of new species and subspecies have been published [2–5]. The history of the classification of peonies first appeared in 1804 and was proposed by an Englishman, H. Andrew. Noteworthy in the history of section *Moutan* classification is the work of Stern in 1946. He accepted Lynch's classification of the entire genus into three sections and they are Section *Moutan*, Section *Paeonia* and Section *Onaepia*. [6] A new classification system for *Paeonia* Sect. *Moutan* was proposed by Hong and Pan [5]. The nine wild tree peonies are divided into subsect. *Vaginatae* and subsect. *Delavayanae* according to the texture and shape of the flower disc. The species of subsect. *Vaginatae* has a leathery disc, and the disc completely envelops the tomentose carpels at anthesis. Conversely, the species belongs to subsect. *Delavayanae* has a fleshy disc and the disc envelops only the base of the glabrous carpels [5].

Pollen morphological characteristics are mainly controlled by genes and little affected by the external environment [7]. It has strong genetic conservativeness, stability, and reliability [8]. The morphological characteristics of pollen have evolved and developed during the long-term evolutionary process [9], which is of great significance in the analysis of species taxonomy and interspecific relatedness [10–12]. The type of pollen aperture can provide theoretical support for the separation of Paeoniaceae plants from Ranunculaceae in terms of palynology [13,14]. Pollen types in Ranunculaceae are diverse, and its apertures types are tricolpate, pantoporate, and stephanocolpate [15], which can be used as the main characteristics to distinguish Paeoniaceae and Ranunculaceae [14]. The species of subsect. *Vaginatae* and subsect. *Delavayanae* have minimal interspecific differences in their pollen morphology under light microscopy, and their aperture types are all of one type, 3-colporoidate [13]. Pollen exine ornamentation is polymorphic, and the taxonomic and evolutionary order among species in tree peony species can be determined based on the types of pollen exine ornamentation [10,16].

Pollen viability involves the ability of pollen to survive and germinate, and the measurement of pollen viability is generally employed to determine whether pollen is capable of fertilization [17]. The pollen viability of different tree peony species or populations is significantly different, which provides information for subsequent pollination [18]. At present, the determination of pollen viability is usually carried out using staining and in vitro germination [19].

Currently, the characteristics of tree peony pollen morphology have been established [10]. However, the classification of these indicators is so vague that a conclusion about the most critical distinction indicators is hard to be made. Meanwhile, the pollen viability of different populations of nine wild tree peony species has rarely been measured systematically and uniformly under the same conditions. In this study, pollen morphology and pollen viability were measured and analyzed using multivariate algorithms to investigate whether one or more pollen indicators could be used to classify the nine wild peony species.

2. Materials and Methods

2.1. Plant Material

Pollen samples from 18 populations of nine tree peony species were collected from mature stamens of living plants cultivated in the Northwest A&F University Germplasm Resource Nursery (108.07° E, 34.26° N). The original habitats of 18 populations are listed in Table 1, and the taxonomic classification (Figure 1) was carried out by Professor Yan-long Zhang. All the samples were taken from well-grown twigs free from pests or diseases.

Table 1. Geographic conditions of the original habitat of nine tree peony species.

Species	Population	Geographic Origin	Longitude E (°) and Latitude N (°)	Elevation (m)
P. rockii	P1	Taibai County, Shaanxi	107.52/33.82	1438
P. rockii	P2	Wudu District, Gansu	105.24/33.32	2202
P. rockii	P3	Mei Co., Shaanxi	107.70/34.08	1528
P. rockii	P4	Feng Co., Shaanxi	106.46/33.94	1386
P. rockii	P5	Liuba Co., Shaanxi	107.15/33.75	1250
P. rockii	P6	Ganquan Co., Shaanxi	106.96/36.58	1412
P. ostii	P7	Mei Co., Shaanxi	107.71/34.07	1563
P. ostii	P8	Shangnan Co., Shaanxi	110.85/33.53	653
P. qiui	P9	Shennongjia, Hubei	110.67/31.77	1976
P. qiui	P10	Xunyang Co., Shaanxi	109.32/32.98	1558
P. qiui	P11	Shangnan Co., Shaanxi	110.63/33.40	1141
P. jishanensis	P12	Jishan Co., Shanxi	110.95/35.72	1163
P. decomposita	P13	Maerkang Co., Sichuan	102.02/32.00	2504
P. decomposita	P14	Li Co., Sichuan	103.45/31.58	2176
P. delavayi	P15	Lijiang City, Yunnan	100.17/26.80	3015
P. potaninii	P16	Yajiang Co., Sichuan	101.15/30.07	3127
P. lutea	P17	Chayu Co., Tibet	96.79/28.72	2980
P. ludlowii	P18	Linzhi City, Tibet	94.63/29.48	2958

Figure 1. Flowers of nine tree peony species from 18 populations. Population codes refer to Table 1.

2.2. Pollen Morphology

The anthers collected from unopened flowers were placed in Petri dishes and dried out at room temperature (24 h) under an incandescent lamp until the pollen was released. Pollens were collected in a self-sealing bag and stored at 4 °C in a desiccator containing silica gel until scanning electron microscopy analysis [20]. Pollen grains were mounted by a fine brush on aluminum stubs covered with double-sided transparent tape and coated with a 0.02 μm thick gold layer on a sputter coater. Observations were carried out under a Hitachi S-3400N scanning electron microscope.

The pollen grains (three replicates with 6 grains) from each population were observed and photographed at a magnification of 1500× or 2000× for the whole grain and 8000× for the exine pattern. The following characteristics were measured and determined: polar length (P), equatorial diameter (E), P/E ratios, perforation diameter (D), murus width (W), and D/W ratio. Size, amb, pollen shape, and exine ornamentation type were qualitatively observed and recorded. The pollen size was classified as "very small" (<10 μm), "small" (10–25 μm), "medium" (26–50 μm), "large" (51–100 μm), and "very large" (>100 μm) depending on the value of equatorial diameter. Polar length (P): equatorial diameter (E) ratios were determined and the shape classes were defined [21].

2.3. Pollen Viability

Pollen viability was tested by pollen germination in vitro [19] on a medium kept at 25 °C. The medium is composed of 0.05 g/L boric acid, 0.04 g/L calcium chloride, 100 g/L sucrose, and 0.02 g/mL agar. The pollen germination rate and pollen tube growth process were evaluated under a light microscope after a 3 h incubation period. Of them, only the pollen grains with pollen tubes equal to or longer than the diameters of the pollen themselves were considered to be germinated [22]. To determine the germination rates of the populations, at least three visual fields were randomly selected.

2.4. Statistical Analysis

Principal component analysis (PCA) and cluster analysis were carried out for the classification of the tree peony populations based on pollen characteristics. For each quantitative characteristic, the mean values were calculated. Qualitative characteristics were analyzed using quantizing ordered polymorphic variables. For the polymorphic values, the polymorphic characteristics were divided into several grades and then encoded according to grades [23]. The specific codes were listed in Table 2. PCA was performed with SPSS version 26.0 for Microsoft Windows. Descriptive palynological terminology follows the protocols of Erdtman [24] and Punt [25]. To clarify the relationship between populations, cluster analysis was performed by OriginPro 2022 software. From which, a dendrogram was developed in terms of Euclidean distance based on the data of nine indices (P, E, P/E, D, W, D/W, amb, equatorial view, exine ornamentation).

Table 2. The specific codes of shape classes, amb, and exine ornamentation.

Code	Shape	Amb	Exine Ornamentation
1	Perprolate	Three-lobed circular	Reticulate
2	Prolate	Obtuse triangular	Microreticulate
3	-	-	Foveolate
4	-	-	Rugulate–reticulate

3. Results

3.1. Morphology of Pollen Grains

All the pollen morphological data examined in this study are listed in Table 3. The results showed similarities and variabilities that occurred in different species or populations in the same species. The outline of pollen can be classified as obtuse triangular, three-lobed circular in the polar view, and the shape classes were perprolate or prolate (Figure 2). Significant differences in the length of the polar and equatorial axes of pollen grains were observed in different populations. The poll ranged from 19.54 to 23.76 in mean equatorial diameter, and it varied from 39.69 to 49.25 in polar length. Therefore, the species ranged from small to medium in size. The ratio of the polar length (P) to the equatorial length (E) for the different populations varied from 1.94 to 2.25 (Table 3).

Table 3. Pollen micromorphological features of the examined population.

Subsection	Species	Population	P/μm	E/μm	P/E	D/μm	W/μm	D/W	Pollen Shape	Amb	Exine Ornamentation
subsect. *Vaginatae*	*P. rockii*	P1	48.85	21.72	2.25	1.07	0.52	2.04	Perprolate	Three-lobed circular	Reticulate
	P. rockii	P2	44.18	22.72	1.94	1.82	0.33	5.48	Prolate	Three-lobed circular	Reticulate
	P. rockii	P3	44.18	22.34	1.98	1.15	0.45	2.58	Prolate	Three-lobed circular	Reticulate
	P. rockii	P4	47.04	23.76	1.98	1.67	0.66	2.51	Prolate	Three-lobed circular	Reticulate
	P. rockii	P5	44.81	19.8	2.26	1.44	0.45	3.2	Perprolate	Three-lobed circular	Reticulate
	P. rockii	P6	45.83	23.05	1.99	1.14	0.58	1.97	Prolate	Obtuse triangular	Reticulate
	P. ostii	P7	44.96	20.02	2.25	0.59	0.53	1.11	Perprolate	Three-lobed circular	Microreticulate
	P. ostii	P8	49.25	21.67	2.27	0.71	0.43	1.65	Perprolate	Three-lobed circular	Microreticulate
	P. qiui	P9	48.15	22.28	1.75	0.89	0.54	1.02	Perprolate	Three-lobed circular	Microreticulate
	P. qiui	P10	45.11	19.54	2.31	0.52	0.5	1.04	Perprolate	Three-lobed circular	Foveolate
	P. qiui	P11	46.85	21.4	2.19	0.62	0.43	1.44	Perprolate	Three-lobed circular	Foveolate
	P. jishanensis	P12	47.51	22.06	2.15	1.78	1.22	1.46	Perprolate	Three-lobed circular	Foveolate
	P. decomposita	P13	47.18	22.58	2.09	0.71	0.53	1.35	Perprolate	Three-lobed circular	Reticulate
	P. decomposita	P14	46.11	22.64	2.04	0.22	0.61	0.36	Perprolate	Three-lobed circular	Foveolate
subsect. *Delavayanae*	*P. delavayi*	P15	42.27	20.57	2.05	0.55	0.48	1.15	Perprolate	Three-lobed circular	Rugulate–reticulate
	P. potaninii	P16	39.69	21.5	1.85	0.43	0.46	0.95	Perprolate	Three-lobed circular	Foveolate
	P. lutea	P17	42.58	21.06	2.02	0.37	0.43	0.86	Perprolate	Three-lobed circular	Foveolate
	P. ludlowii	P18	41.37	20.22	2.05	0.39	0.35	1.11	Perprolate	Three-lobed circular	Foveolate

P, polar length; E, equatorial length; D, perforation diameter; W, murus width.

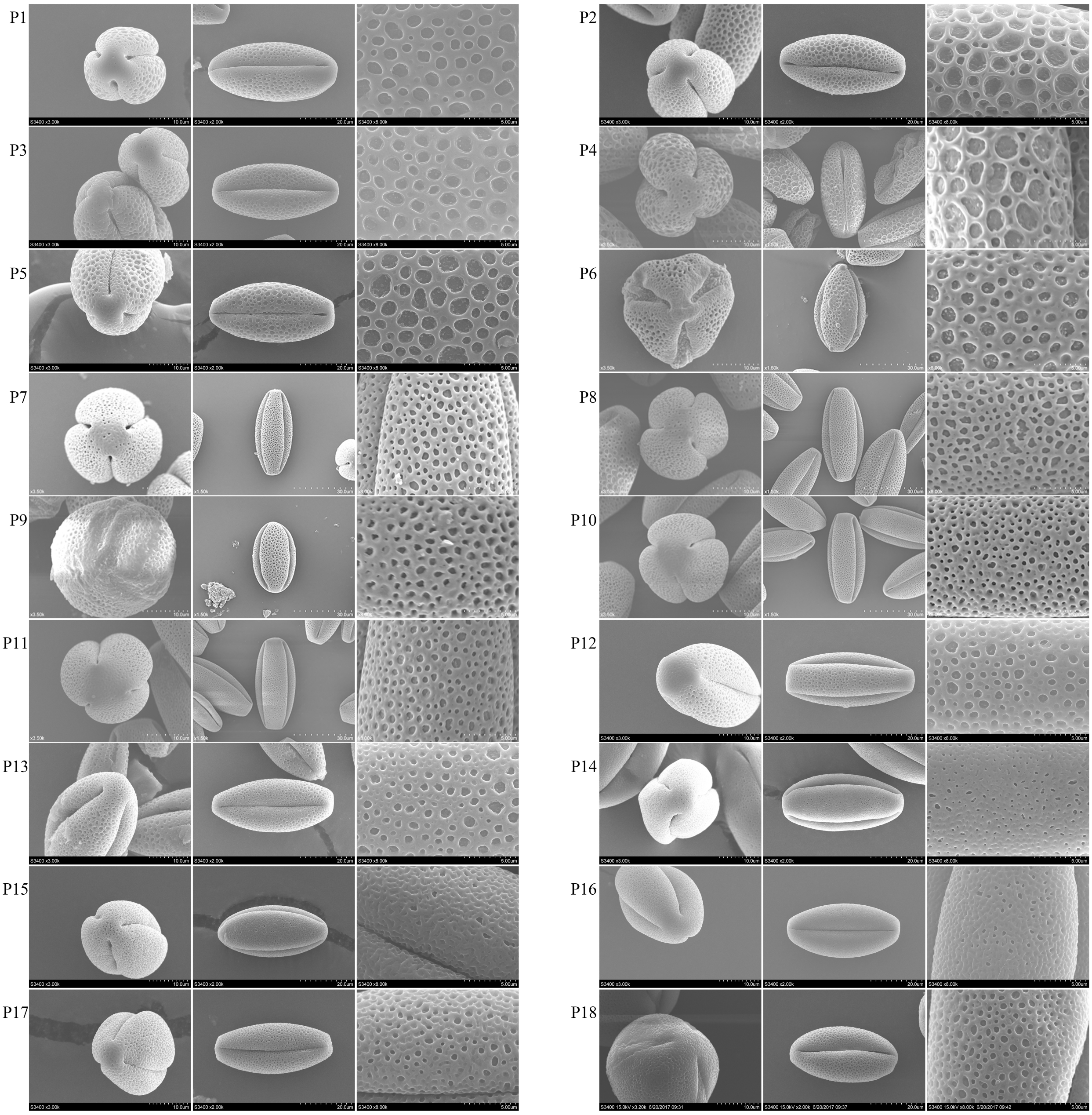

Figure 2. Scanning electron micrographs of pollen grains on 18 tree peony populations. Population codes refer to Table 1.

The pollen grains are monads and 3-colporoidate. The germination ditch extended to the two opposite poles of pollen that was dehiscent. The perforation was the largest in the middle of the equatorial plane and decreased toward the poles and edges. The exine ornamentation of 18 populations was presented as reticulate, microreticulate, foveolate, and rugulate–reticulate. The exine architecture of mature pollen grains photographed at 8000× is shown in Figure 2.

3.2. Principal Component Analysis

The PCA results of the pollen morphology of the 18 populations are shown in Table 4. The data demonstrated that there was considerable variation among the populations based on the morphological properties of pollen. The first three PCAs showed 75.035% variation in total, which were 39.722%, 19.288%, and 16.026%, respectively (Figure 3). The significant characteristics included in PC1 were perforation diameter, D/W, equatorial diameter, pollen shape, exine ornamentation, and amb. P/E and polar length were the main variables included in PC2. The murus width was the most essential variable for PC3. We conducted a cluster analysis using pollen morphological data from 18 populations. A cluster diagram was constructed based on data from the nine morphological traits (Figure 4). From this cluster diagram, the 18 populations could be roughly classified into two clusters. Cluster I named as the subsect. *Vaginatae* group consisted of 14 populations from five species: *P. rockii* (P1, P2, P3, P4, P5, P6), *P. ostii* (P7, P8), *P. qiui* (P9, P10, P11), *P. jishanensis* (P12), and *P. decomposita* (P13, P14). Cluster II was composed of four populations named the subsect. *Delavayanae* group from four species: *P. delavayi* (P15), *P. potaninii* (P16), *P. lutea* (P17), and *P. ludlowii* (P18). These results were consistent with the morphological classification of tree peony [26]. Therefore, it was suggested that pollen morphology could be used as a reference for distinguishing two subsections of Section *Moutan* in 18 populations.

Table 4. Nine values and results of the first three principal components (PCAs) analysis for the pollen characteristics in the tree peony population.

Character	PC1	PC2	PC3
Polar length (μm)	0.391	0.723	0.193
Equatorial diameter (μm)	0.765	−0.093	0.458
P/E	−0.375	0.760	−0.391
perforation diameter	0.814	0.328	−0.158
murus width	0.154	0.573	0.649
D/W	0.708	−0.010	−0.622
Pollen shape	0.855	−0.374	0.068
Amb	0.386	−0.178	0.292
Exine ornamentation type	−0.794	−0.143	0.345
Eigenvalue	3.575	1.736	1.442
Variance (%)	39.772%	19.288%	16.026%
Cumulative variance (%)	39.772%	59.009%	75.035%

To reduce variables and explore the possibility of distinguishing two subsections of Section *Moutan* in 18 populations with fewer indicators of pollen morphology, the 18 populations were clustered again, sing three indicators with large principal components loading according to the results of the principal component analysis in Table S1 (Figure 5). The clustering result is shown using the variables of perforation diameter, D/W, equatorial diameter, pollen shape, exine ornamentation, and murus width in PC1 in Figure 5A. The tree peony of 18 different populations can be divided into four groups. Group I included *P. rockii* (P1), *P. ostii* (P7, P8), *P. qiui* (P9, P10, P11), *P. jishanensis* (P12), *P. decomposita* (P13, P14), *P. delavayi* (P15), *P. potaninii* (P16), *P. lutea* (P17), and *P. ludlowii* (P18). Group II consisted of *P. rockii* (P3, P4, P6). Group III consisted of *P. rockii* (P5). Group IV consisted of *P. rockii* (P2). The P/E and polar length were the variables included in PC2, and the clustering result is displayed in Figure 5B. The tree peony of 18 different populations can be divided into two groups. Group I was subsect. *Vaginatae*, which consisted of *rockii* (P1, P2, P3, P4, P5, P6), *P. ostii* (P7, P8), *P. qiui* (P9, P10, and P11), *P. jishanensis* (P12), and *P. decomposita* (P13, P14). Group II was subsect. *Delavayanae*, which included *P. delavayi* (P15), *P. potaninii* (P16), *P. lutea* (P17), and *P. ludlowii* (P18). The clustering result is presented in Figure 5C with the amb variable for PC3. The tree peony of 18 different populations can be divided into three groups. Group I consisted of *P. rockii* (P1, P3, P4, P5, P6), *P. ostii* (P7, P8), *P. qiui* (P9, P10, P11), *P. decomposita* (P13, P14). *P. delavayi* (P15), *P. potaninii* (P16), *P. lutea* (P17). Group II consisted of *P. rockii* (P2) and *P. ludlowii* (P18). Group III consisted of *P. jishanensis* (P12).

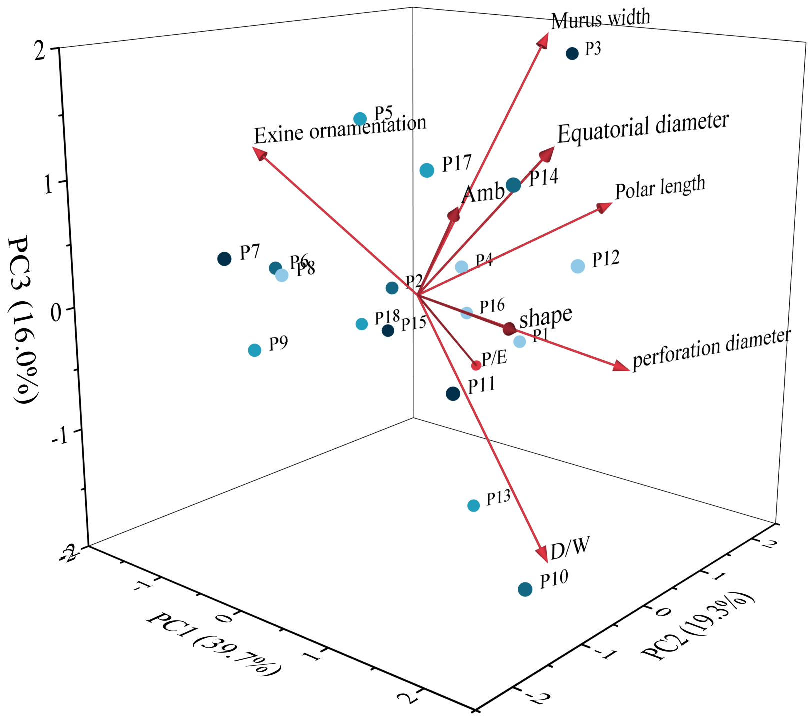

Figure 3. 3D−plot of the principal component analysis (PCA) for pollen characteristics of 18 populations.

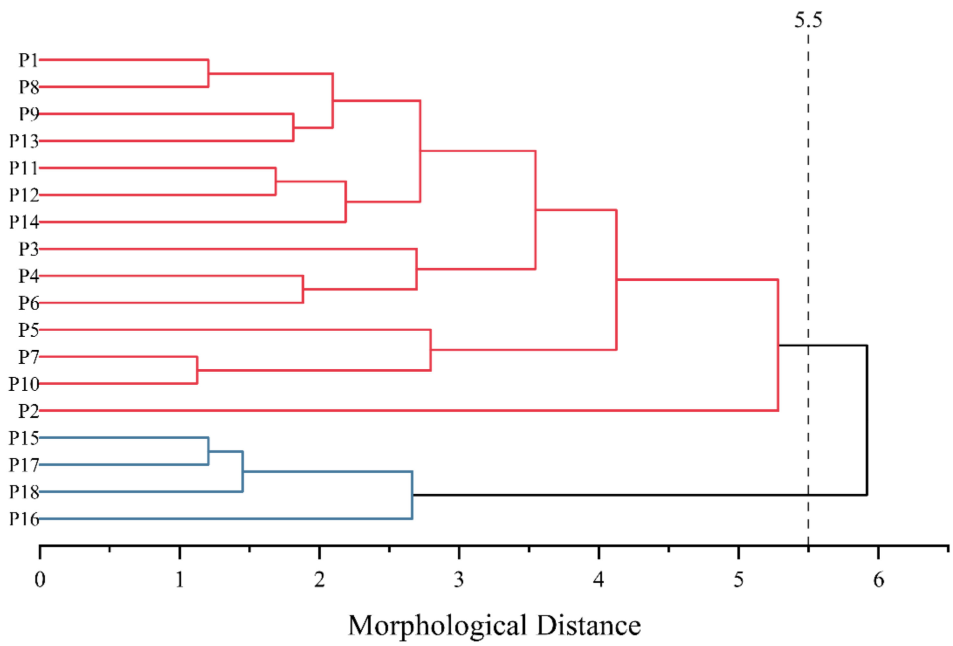

Figure 4. Cluster diagram of 18 tree peony populations based on pollen characteristics. The red line indicates subsect. *Vaginatae* and the blue line indicates subsect. *Delavayanae*.

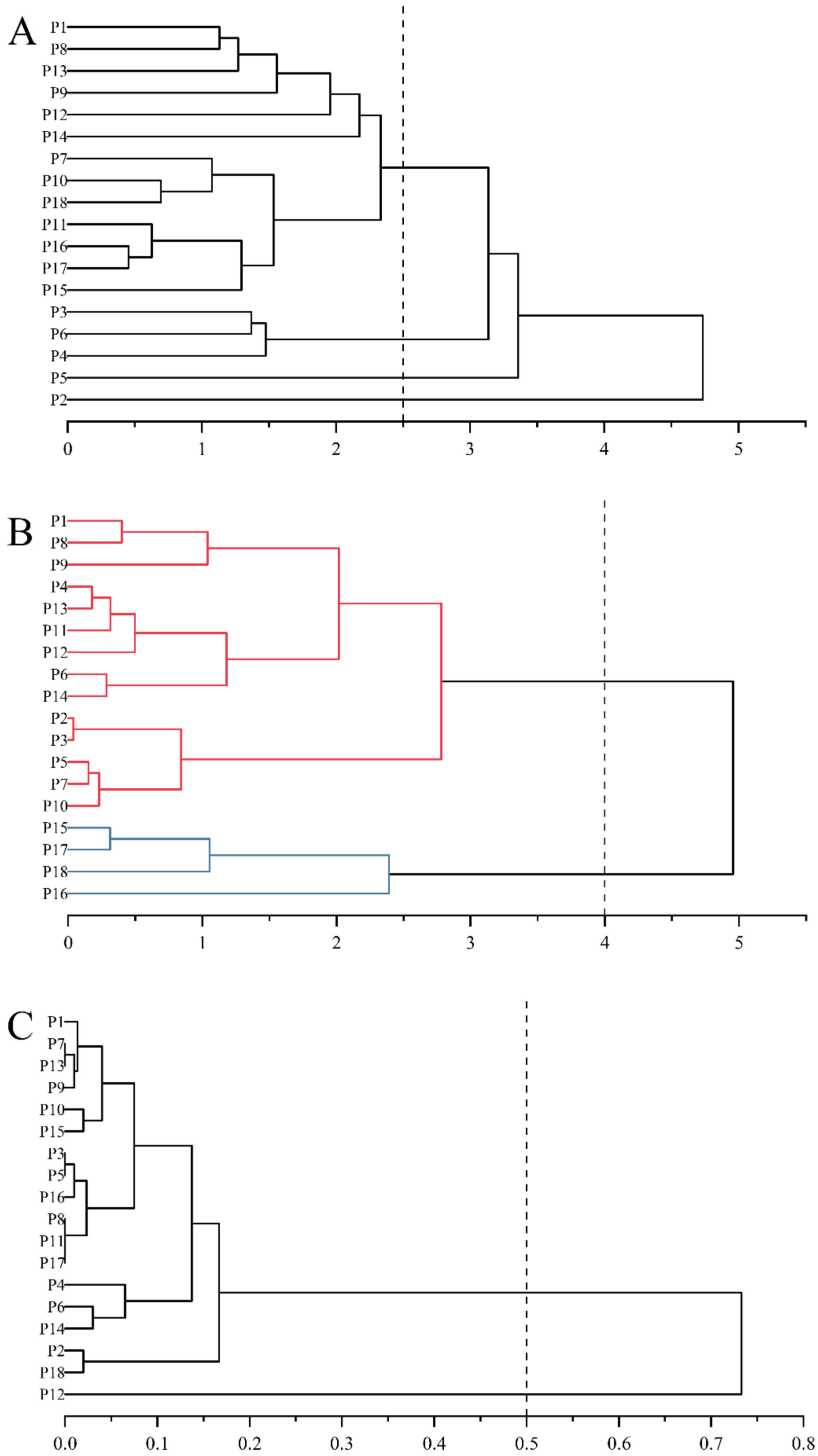

Figure 5. Cluster analysis of 18 tree peony populations based on PC1 (**A**), PC2 (**B**), and PC3 (**C**), respectively. The red line indicates subsect. *Vaginatae* and the blue line indicates subsect. *Delavayanae*.

Through the comprehensive ranking of nine indicators in terms of contribution to variation, we found that the polar length is the first significant factor in distinguishing populations (Table S1). Further cluster analysis showed that 18 populations could be divided into two groups when the Euclidean distance was five with the polar length as the variable (Figure 6). Here, the placement of the taxa in two clusters was exactly similar with that of Figure 4, which indicated that polar length was the key characteristic to distinguishing the two subsections of Section *Moutan*.

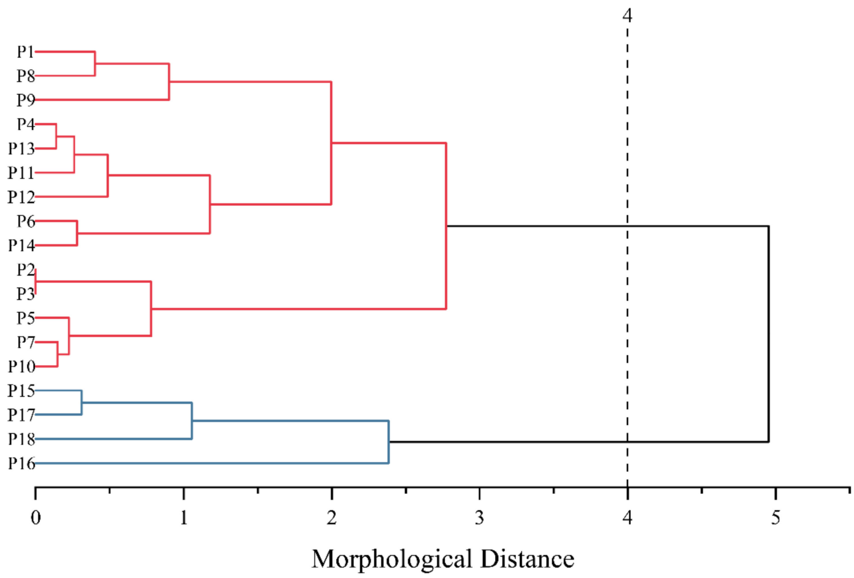

Figure 6. Cluster analysis of 18 tree peony populations based on the pollen polar length. The red line indicates subsect. *Vaginatae* and the blue line indicates subsect. *Delavayanae*.

3.3. Pollen Viability and Germination

To figure out the connection between pollen viability and morphology, pollen germination of all the populations of tree peony were observed under light microscope (Figure S1). The pollen germination rates of different peony in vitro are shown in Table 5. The pollen germination rate of subsect. *Vaginatae* was significantly higher than that of subsect. *Delavayanae*. The average pollen germination rate of 14 populations in subsect. *Vaginatae* was 61.41%, with a maximum of 87.78% (*P. qiui* in Xunyang County, Shaanxi) and a minimum of 20.15% (*P. rockii* in Liuba County, Shaanxi Province); the pollen germination rate of four populations in subsect. *Delavayanae* was 35.41%, with a maximum of 46.34% (*P. lutea* Chayu County, Tibet) and a minimum of 25.77% (P. *delavayi* in Lijiang County, Yunnan Province).

Table 5. The germination rate of different peony pollen in vitro.

Subsection	Population	Pollen Germination Rate (%)	Average (%)
subsect. *Vaginatae*	P1	84.30	61.41
	P2	64.17	
	P3	80.02	
	P4	77.29	
	P5	20.15	
	P6	26.16	
	P7	66.63	
	P8	73.61	
	P9	48.19	
	P10	87.78	
	P11	61.04	
	P12	62.62	
	P13	56.88	
	P14	50.89	
subsect. *Delavayanae*	P15	25.77	35.41
	P16	36.61	
	P17	46.34	
	P18	32.91	

4. Discussion

4.1. Morphology of Pollen Grains

The pollen morphological characteristics of 18 populations in the section *Moutan* DC. were investigated in this study. The outline of pollen is obtuse triangular and three-lobed circular in the polar view, and the shapes of pollens are prolate and perprolate, which was approximately consistent with the previous study [13]. The pollen grains of tree peony are similar in size and shape to those of herbaceous peony [27]. Pollen grains of the genus *Paeonia* L. are in the structure of covering layer-columnar-layer with the apertures types of 3-colporoidate [13].

The significant differences in the exine ornamentation of pollen among different tree peonies indicate that genetic diversity is abundant among tree peonies. The pollen exine ornamentation can be divided into four types in previous studies, including micro-foveolate, foveolate, reticulate, and reticulate (with coarse reticulum) based on the evolutionary route. In the evolutionary process of wild peony, the evolution of leaflets is likely to proceed from entire leaf to divided leaf, provided that the basic leaf shape remains unchanged, which indicates that the evolutionary rules of morphological characteristics and exine ornamentation are basically consistent [10]. In addition, the pollen exine ornamentation can also be divided into striate–reticulate, rugulate–reticulate, and rugulate [28]. In our study, four types of pollen exine ornamentation appeared in 18 populations, reticulate, microreticulate, foveolate and rugulate–reticulate. Among them, it is surprising that foveolate is observed for *P. jishanensis* in Jishan County, Shanxi province, which has not been reported in previous studies. The pollen exine ornamentation of *P. jishanensis* is rugulate and reticulate [28]; hence, we hypothesize that it may be the result of the evolution of the species. In addition to the differences that occurred in species, significant differences also can be found in different populations of the same species. In this study, the pollen exine ornamentation of *P. ostii* is mainly classified into two types: reticulate (P3, P5) and reticulate (with coarse reticulum) (P1, P2, P4, P6). Nevertheless, its exine ornamentation is foveolate whether it is planted in Luoyang City, Henan province, or Tongling and Bozhou, Anhui province [29]. The pollen exine ornamentation of *P. qiui* from three populations has been described to be evolved from the primitive foveolate to the rugulate. Based on this hypothesis, the evolutionary order of these three populations was inferred to be: Xunyang and Shennongjia, and finally to Shangnan County, which was consistent with the evolutionary route reported [10]. Meanwhile, this also suggests that palynology is informative in terms of systematic classification and species identification.

4.2. Principal Component Analysis

The specificity of pollen morphological characteristics plays a vital role in the taxonomic classifications and genetic relationships. It is suggested that the differences in pollen morphology among different species can be used as a reference for plant classification [11,30]. In previous studies, the characteristics of tree peony pollen grain morphology were identified, and the relationship between evolutionary trends and morphological characteristics was also investigated by cluster analysis [10]. From their analysis, five species in subsect. *Vaginatae* were clearly divided into *P. qiui* and *P. jishanensis* had the closest genetic relationship, and so did *P. ostii* and *P. rockii. P. decomposita* has a relatively distant genetic relationship with the other species [10]. The pollen morphologies of Japanese cultivars and Chinese central plains cultivars were greatly different from that of the northwest cultivars, and cluster analysis further demonstrated the genetic relationship between Chinese central plains cultivars and Japanese cultivars was closer [31]. It showed that the morphological characteristics of pollen could also be used as a vital reference for cultivar classification. The cluster analysis of the pollen morphology of 26 herbaceous peony cultivars with different ploidy levels was carried out by six characteristics, which provided a functional palynological foundation for studying kinship and taxonomy among peonies [32]. However, no research has been reported to quantify the indicators through specific algorithms to refine the classification foundation.

In this study, we intended to further determine the classification characteristics through the specific data foundation. Therefore, it would be unsurprising that Figure 5A will be the most similar cluster to that of Figure 4 if the clustering was carried out according to the contribution rate of principal components. However, the analysis was contrary to the inference. Our findings confirmed that Figure 5B was the highest similar cluster to Figure 4. The discrepancy could be attributed to the failure to rank the nine pollen morphology characteristics comprehensively. The contribution in variation of the polar length ranked first in the PCA by up to 22.4%. The reason for the similarity between Figures 5B and 4 may be that Figure 5B contains the highest weighted morphological characteristics polar length. Therefore, we performed the cluster analysis again based on the polar length (Figure 6). The 18 populations were divided into two groups: subsect. *Vaginatae* and subsect. *Delavayanae*, which was consistent with the previous morphological classification of tree peony [26]. The polar length in pollen morphology is further taken as an essential foundation to distinguish the two subsections based on the specific quantitative data. The pollen polar lengths of five wild species in subsect. *Vaginatae* are longer than 43 µm, and that of four wild species in subsect. *Delavayanae* are shorter than 43 µm.

In addition, cluster analysis of 18 populations showed that populations could not be completely classified according to geographical distance, which is consistent with the results of chloroplast DNA analysis in 20 populations of *P. rockii.* [33]. Moreover, populations from one species were not completely clustered together, which was especially noticeable in *P. rockii*. This result resembles the clustering results of 27 populations from 5 wild species in subsect. *Vaginatae* [34]. The reason for this is that poor clustering was generated due to few pollen morphology data. As a result, it is necessary to add much more representative indicators in future work, such as the length and width of the germinal furrow, the glossiness of lirae, aperture shape and size, and aperture prominence.

4.3. Pollen Viability and Germination

To determine the pollen viability, we adopted the germination in vitro determination method because of its accuracy under a microscope [35]. Although the staining method is rapid, other than normal pollen, the immature, aging, and abortive pollen can also be stained. As the result of massive staining, the value measured for the pollen viability could not be used to represent the actual level correctly [36,37]. Our results showed that the pollen viability of the subsect. *Vaginatae* and subsect. *Delavayanae* varied significantly. The pollen germination rates in subsect. *Vaginatae* are higher than in subsect. *Delavayanae*. Pollen viability in subsect. *Vaginatae* are basically higher than 50%, while all in subsect. *Delavayanae* are lower than 50%. The above results can serve as additional proof that pollen characteristics can be used as a basis for the classification of tree peonies. In addition, large differences also occur in the same species. For example, the pollen germination rate of *P. rockii* varied from 20.15% to 84.3%. The wide range of ecological adaptations and different environmental conditions and habitat types make subsect. *Vaginatae* of tree peonies are rich in morphological traits and genetic variation, especially the variation of floral traits within different populations and populations of *P. rockii* [38]. Similar differences in pollen viability were observed in pear [39] and jujube [40].

Pollination can be simply predicted by the level of pollen viability. The key to efficient cross-breeding lies in the scientific selection of parents according to the genetic principle of traits [41]. Based on the clustering results of pollen morphology and the pollen germination rate, the genetic relationship between *P. qiui* (Xunyang County, Shanxi) and *P. ostii* (Mei County, Shaanxi) is close, and they have higher pollen germination rates. Our findings are consistent with the conclusion that there is higher cross-compatibility between *P. qiui* and *P. ostii* [42]. There will be more combinations theoretically feasible to do the cross based on this principle: *P. rockii* (Taibai County, Shaanxi Province), *P. ostii* (Shangnan County, Shaanxi Province), *P. qiui* (Shangnan County, Shaanxi Province), and *P. decomposita* (Maerkang, Sichuan Province). These predicted combinations have been proven to be practicable in the other study. *P. ostii* and *P. qiui* are both excellent parent materials with strong fertility and are better as female parents, and *P. decomposita* is better as a male parent [43].

5. Conclusions

The present study aimed to identify a crucial pollen characteristic to distinguish tree peony using multivariate analysis based on pollen morphology and pollen viability. The most prominent finding from this study is that the polar length in pollen morphology is a key characteristic for distinguishing the two subsections. The pollen polar length of five wild species in subsect. *Vaginatae* is longer than 43 µm; however, that of four wild species in subsect. *Delavayanae* is shorter than 43 µm. Additionally, pollen germination rates in subsect. *Vaginatae* are significantly greater than in subsect. *Delavayanae*. All pollen germination rates in subsect. *Vaginatae* are basically more than 50%, while all in subsect. *Delavayanae* are less than 50%. In summary, this study provided a new taxonomic idea for the intersectional classification of tree peonies in terms of pollen morphology and pollen viability and determined the final classification index of pollen characteristics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8080736/s1. Figure S1: Light micrographs of pollen grain germination in vitro of 18 populations. Population codes refer to Table 1. Table S1: Correlation coefficient (*r*) values of the pollen characteristics of 18 populations.

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