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The morphological diversity of pollen in the genus *Gossypium*

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Abstract

Background Plant pollen has diverse morphological characteristics that can be consistently passed down from generation to generation. Information on pollen morphology is thus immensely important for plant classification and identification. In the genus *Gossypium*, however, in-depth research on pollen morphology is lacking, with only few reports on limited cotton species. To evaluate the diversity of pollen in *Gossypium*, we therefore conducted a comprehensive analysis of the pollen morphology of 33 cotton species and varieties using scanning electron microscopy.

Results The 33 analyzed cotton samples exhibited common pollen morphological features, including spherical shapes, radial symmetry, echination, panporation, and operculation, while the pollen size, spine shape, spine density and length showed distinctive features. Pollen size varied significantly among species, with diameters ranging from 62.43 μm in *G. harknessii* to 103.41 μm in *G. barbadense*. The exine had an echinate sculptural texture, and spines were mostly conical or sharply conical but occasionally rod-like. Spine density varied from 173 in *G. incanum* to 54 in *G. gossypioides*, while spine length ranged from 3.53 μm in *G. herbaceum* to 9.47 μm in *G. barbadense*. In addition, the 33 cotton species and varieties were grouped at a genetic distance of 3.83 into three clusters. Cluster I comprised five allotetraploid AD-genome cotton species, four D-genome species, and one K-genome species. Cluster II included 13 diploid species from A, B, D, E, and G genomes, whereas Cluster III only consisted one E-genome species *G. incanum*.

Conclusions Although pollen characteristics alone are not enough to resolve taxonomic and systematic relationships within the genus *Gossypium*, our results add to knowledge on palynomorphology and contribute to phenological information on these taxa. Our findings should aid future systematic and phylogenetic studies of the *Gossypium* genus.

Keywords Cotton, Scanning electron microscopy, Pollen morphology, Diversity

Introduction

Gossypium L. (cotton), the largest and most widely distributed genus in the Gossypieae, comprises more than 50 species (Fryxell 1992), including 46 diploid species ($2n = 2x = 26$) as well as 5 well-established and 2 newly identified tetraploid species ($2n = 4x = 52$) (Grover et al. 2014; Wendel and Grover 2015; Wang et al. 2018). The diploid cotton species are believed to have originated from a common ancestor approximately 5 million to 10 million years ago (MYA) and subsequently diversified cytogenetically into eight genome groups (designated A to G and K) that differ in chromosome

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size but not in number (Wendel and Cronn 2003). *Gossypium* species vary greatly in morphology, ranging from herbaceous perennials to subshrubs and even to small trees. Moreover, the flowers of *Gossypium* also differ in size and color. The AD genome group is predominantly characterized by the presence of ivory-white or bright yellow flowers, whereas rosy flowers are prevalent among species belonging to the G, C, and K genome groups of Australia.

Cotton is an economically significant crop worldwide and also serves as a model species for evolutionary studies (Wendel et al. 2012). An extensive amount of molecular phylogenetic research has been conducted on *Gossypium* (Cronn et al. 2002; Wendel and Cronn 2003; Grover et al. 2008; Xu et al. 2012; Wendel and Grover 2015; Hu et al. 2021; Wang et al. 2022). For example, the phylogenetic relationships of diploid cotton genome groups have been evaluated using 16 nuclear and chloroplast genes, which revealed that cotton genome groups underwent rapid radiation after formation of the genus (Cronn et al. 2002). Grover et al. (2015) has updated previously assumed connections among *Gossypium* allopolyploids by applying targeted sequence capture of multiple loci in conjunction with both concatenated and Bayesian concordance. The report has discovered that a newly identified allopolyploid species (*G. ekmanianum*) is closely related to *G. hirsutum* and has established a more robust phylogeny for allopolyploid *Gossypium* (Grover et al. 2015). A comparative analysis of 19 *Gossypium* chloroplast genomes by Chen et al. (2016) has divided the eight diploid genome groups into six clades. The analysis has revealed contrasting evolutionary dynamics in different clades, with parallel genome downsizing in two genome groups and a biased accumulation of insertions in the clade containing cultivated cottons leading to large chloroplast genomes. The rapid global diversification of the *Gossypium* genus has resulted in some challenging phylogenetic questions.

Pollen morphological characteristics can provide a reliable basis for classifying and identifying the origin and evolution of plants (Rosenfeldt and Galati 2007; Erik 2012; Baser et al. 2016; Mezzonato-Pires et al. 2018; Reunov et al. 2018; Grimsson et al. 2019; Zhang et al. 2021; Umber et al. 2022). For example, pollen grains of *Sclerosperma mannii* and *S. walheri* share the same distinct reticulate sculpture, which suggests that these two currently accepted *Sclerosperma* species are sister taxa of the same intrageneric lineage (Grimsson et al. 2019). Pollen grains from different species have developed unique morphological characteristics during evolutionary while many common morphological features has passed on within families and genera. These morphological features are controlled by genes and not easily be affected by external environment, thus can be used in plant taxonomy.

A previous investigation based on pollen wall stripping and scanning electron microscopy (SEM) has revealed that the pollen grain size of *G. barbadense* was the largest among four cultivated cotton species (Jia et al. 1988). Another study has used SEM to examine the ultrastructure of upland cotton (*G. hirsutum*) pollen at various developmental stages and has discovered that the pollen walls are covered with fully grown spiny protuberances and apertures (Liu et al. 1994). Despite these prior investigations of the pollen morphology of some cotton species, no systematic studies have been conducted. In the current study, we therefore performed a comprehensive SEM-based survey of 33 species and varieties of *Gossypium*. Our findings provide detailed knowledge of cotton palynomorphology and have potential value for elucidating cotton taxonomy and evolutionary history.

Materials and methods

Plant materials

A total of 33 germplasm accessions representing 23 *Gossypium* species were evaluated in the present study (Table 1). All specimens were planted at the National Wild Cotton Germplasm Resources Nursery (Sanya, Hainan, China), which is supervised by the Institute of Cotton Research, Chinese Academy of Agricultural Sciences (ICR-CAAS), Anyang, Henan, China. Pollen grains wrapped in anthers were collected in the morning from intact, freshly opened flowers. The grains were fixed in 2.5% glutaraldehyde solution (in 0.2 mol·L^{-1} phosphate buffer; pH 7.4) for 3 h, washed at least three times with 0.135 mol·L^{-1} phosphate buffer ($Na_2HPO_4 \cdot 7H_2O$, 30.40 g; $NaH_2PO_4 \cdot H_2O$, 2.98 g; pH 7.4), soaked in 2.5% glutaraldehyde solution, and stored at 4 °C.

Scanning electron microscopy (SEM) analysis of cotton pollen

Micro-morphological features of cotton pollen were examined by SEM following the protocol of Lan and Xu (1996). Pollen grains were stepwise dehydrated in 50%, 70%, 90%, and 100% ethanol. Treated pollen grains were then mounted onto the surface of polished aluminum stubs using double-sided adhesive tape. Each stub was sputter coated with a gold layer and taped to the object stage. Observations and micrograph acquisition were carried out under a scanning electron microscope (Hitachi S-350) installed at the Institute of Cotton Research, Chinese Academy of Agricultural Sciences. Biometric measurements were conducted using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, Maryland, USA). Measurements were carried out on 20 randomly chosen, mature, non-malformed pollen grains per sample.

Table 1 The information of materials

Number	Taxon	Genome group	Genome size /Mb	Material name	Geographical distribution/origin
1	*Gossypium hirsutum* Linnaeus	AD_1	2 295	*G.hirsutum*(CRI12)	Many parts of the drier New World tropics and subtropics (Wang et al. 2018)
2	*Gossypium barbadense* Linnaeus	AD_2	2 225	*G.barbadense*(CL)	Many parts of the drier New World tropics and subtropics (Wang et al. 2018)
3	*Gossypium barbadense* Linnaeus	AD_2	2 225	*G.barbadense*(YM)	Many parts of the drier New World tropics and subtropics (Wang et al. 2018)
4	*Gossypium barbadense* Linnaeus	AD_2	2 225	*G.barbadense*(XH7)	Many parts of the drier New World tropics and subtropics (Wang et al. 2018)
5	*Gossypium tomentosum* Nuttall ex Seemann	AD_3	2 194	*G.tomentosum* (LZ)	Hawaii Islands, USA
6	*Gossypium tomentosum* Nuttall ex Seemann	AD_3	2 194	*G.tomentosum*(JFZ8)	Hawaii Islands, USA
7	*Gossypium tomentosum* Nuttall ex Seemann	AD_3	2 194	*G.tomentosum*(01)	Hawaii Islands, USA
8	*Gossypium mustelinum* Miers ex Watt	AD_4	2 297	*G.mustelinum*(LZ)	Brazil
9	*Gossypium mustelinum* Miers ex Watt	AD_4	2 297	*G.mustelinum*(16)	Brazil
10	*Gossypium darwinii* Watt	AD_5	2 183	*G.darwinii*(07)	Galapagos Islands
11	*Gossypium herbaceum* Linnaeus	A_1	1 565	*G.herbaceum*(ZC1)	Africa
12	*Gossypium herbaceum subs. africanum* Hutchinson	$A_{1\text{-}a}$	1 556	*G.herbaceum* (africanum)	Southern Africa
13	*Gossypium arboreum* Linnaeus	A_2	1 694	*G.arboreum* (SXY)	Asia
14	*Gossypium arboreum* Linnaeus	A_2	1 694	*G.arboreum*(Rozi)	Asia
15	*Gossypium anomalum* Wawra & Peyritsch	B_1	1 231	*G.anomalum*(LZ)	Africa
16	*Gossypium capitis-viridis* Mauer	B_3	1 140	*G.capitis-viridis*(LZ)	Cape Verde Islands
17	*Gossypium capitis-viridis* Mauer	B_3	1 140	*G.capitis-viridis*(01)	Cape Verde Islands
18	*Gossypium thurberi* Todaro	D_1	750	*G.thurberi*(LZ)	Arizona,USA
19	*Gossypium thurberi* Todaro	D_1	750	*G.thurberi*(35)	Arizona,USA
20	*Gossypium armourianum* Kearney	$D_{2\text{-}1}$	756	*G.armourianum*(LZ)	Mexica and California
21	*Gossypium harknessii* Brandegee	$D_{2\text{-}2}$	750	*G.harknessii* (LZ)	Mexica and California
22	*Gossypium davidsonii* Kellogg	$D_{3\text{-}d}$	838	*G.davidsonii* (LZ)	Mexica and California
23	*Gossypium klotzschianum* Andersson	$D_{3\text{-}k}$	671	*G.klotzschianum* (LZ)	Galapagos Islands
24	*Gossypium raimondii* Ulbrich	D_5	775	*G.raimondii*(01)	Peru
25	*Gossypium gossypioides*(Ulbrich)Standley	D_6	761	*G.gossypioides*(LZ)	Oaxaca, Mexica
26	*Gossypium schwendimanii* Fryxell & Koch	D_{11}	729	*G.schwendimanii*(LZ)	Mexica
27	*Gossypium stocksii* Masters in Hooker	E_1	1 501	*G.stocksii* (LZ)	Arabia
28	*Gossypium somalense* (Gurke) Hutchinson	E_2	1 370	*G.somalense*(LZ)	North Africa
29	*Gossypium areysianum* Deflers	E_3	1 402	*G.areysianum* (LZ)	South Yemen
30	*Gossypium incanum* (Schwartz) Hillcoat	E_4	1 491	*G.incanum* (04)	South Africa
31	*Gossypium bickii* Prokhanov	G_1	1 609	*G.bickii*(LZ)	Central Australia
32	*Gossypium australe* Mueller	G_2	1 606	*G.australe* (LZ)	Australia
33	*Gossypium rotundifolium* Fryxell, Craven & Stewart	K_2	2 435	*G.rotundifolium* (LZ)	Northwest Australia

Data exploration and statistical analysis

In this study, we measured three quantitative pollen characters of at least 20 pollen grains per collected specimen, including pollen diameter, exine spine length, and exine spine density (the number of spine on the front side of each pollen grain). We also examined the following qualitative characters: pollen shape, spine type, and protuberance at the base of exine spine. Protuberances were classified into three categories: small, medium, and significant. All pollen terminology follows the glossary in Erdtman (1987) and Punt et al. (2007). All statistical analyses were conducted using SPSS18.0 software. Interrelationships of the six evaluated traits were analyzed based on Pearson correlation coefficients. A euclidean distance matrix of the standardized data was then subjected to cluster analysis by the unweighted pair group method with arithmetic mean as implemented in NTSYS pc version 2.1.

Results

Pollen morphology

The results of SEM analysis of the pollen morphology of 33 cotton specimens are shown in Fig. 1 and Table 2. We found that pollen grains of all examined samples were spherical, radially symmetrical, and panporate. Each pollen grain had more than eight nearly operculate apertures. Aperture number varied widely among cotton taxa, with the largest number present in *G. gossypioides* (Fig. 1y). In contrast, little variation was observed in pollen shape, which was spheroidal in all *Gossypium* species.

Pollen size is normally calculated as the product of polar and equatorial diameters. Because the pollen grains of all examined cotton species were spheroidal, however, polar and equatorial views could not be easily distinguished. We therefore used pollen diameter to represent pollen size. On this basis, the size of pollen grains generally varied widely among cotton species. The largest mean pollen diameter among 33 samples was in *G. barbadense*, 103.41 μm, and the smallest mean value was in *G. harknessii*, 62.43 μm (Fig. 2A). In a previous study, the largest pollen grains were those of island cotton (*G. barbadense*), followed by upland cotton (*G. hirsutum*), *G. arboreum*, and *G. herbaceum* (Jia et al. 1988). We also found that pollen grains of *G. barbadense* were significantly larger than those of other cotton species. Moreover, we discovered that pollen grains of allotetraploid (AD genome) cotton species, with a mean pollen diameter of 94.7 μm, were much larger than those of diploid cotton species (A–K genomes), which showed mean pollen diameters in the range of 69.66 μm (G genome) to 87.25 μm (K genome) (Fig. 2B). Pearson correlation analysis of cotton genome size (Table 1) and pollen size (Table 2) yielded an r value of 0.648, which indicates a moderately positive correlation between these two characters. Detailed results are listed in Table 3.

Exine sculpture

We observed a large variation in the shape, number, and length of exine spines within the genus *Gossypium*. Spine shape was categorized as sharply conical, conical, or rod-like based on the sharp top of spines (Table 2). Accordingly, we uncovered significant differences among the studied species: 15 of sharply conical, 14 of conical and 3 of rod-like. The rod-like type of exine spine was rarely present in *G. harknessii* or E-genome species *G. somalense* or *G. areysianum*. In addition, the size of the protuberance at the base of exine spine, which was classified as small, medium, or obvious, varied among samples (Table 1).

Spine density varied from 54 in *G. gossypioides* to 173 in *G. incanum*. Large variations in spine density were observed between cotton species, whereas intraspecific differences were smaller. Exine spine numbers in three varieties of *G. tomentosum* (AD_3) were 76, 64, and 57, and the number of *G. mustelinum* (AD_4) were 89 and 87. Notably, the number of spines in *G. incanum* (173) was higher than that of other E-genome species, such as *G. stocksii* (118), *G. somalense* (100), and *G. areysianum* (106).

Spine lengths in different species ranged from 3.53 μm in *G. herbaceum* to 9.47 μm in *G. barbadense*, which was consistent with the measurements of pollen size (Fig. 3A). Similarly, the mean exine spine length of AD-genome species was larger than that of all diploid species except for K-genome member *G. rotundifolium* (Fig. 3B). Among allotetraploids (AD genome), the spine length of *G. hirsutum* (AD_1) was similar to that of *G. tomentosum* (AD_3), whereas the spine length of *G. barbadense* (AD_2) was nearly the same as that of *G. darwinii* (AD_4). These results suggested a close relationship of *G. hirsutum* versus *G. tomentosum* and of *G. barbadense* (AD_2) versus *G. darwinii* (AD_4), consistent with scientifically recognized phylogenetic classifications. A Pearson correlation analysis was performed to examine the relationship between pollen diameter and spine length. The Pearson correlation coefficient was found to be 0.690, indicating a significant correlation between these two variables (Table 4).

Cluster analysis

A cluster analysis was conducted using the morphometric data obtained from 33 samples of 23 *Gossypium* species (Fig. 4). At a genetic distance of 3.83, samples were separated into three clusters. Cluster I consisted of five allotetraploid AD-genome species (*G. hirsutum*, *G. barbadense*, *G. tomentosum*, *G. mustelinum*, and *G. darwinii*) and five diploid species (*G. klotzschianum*,

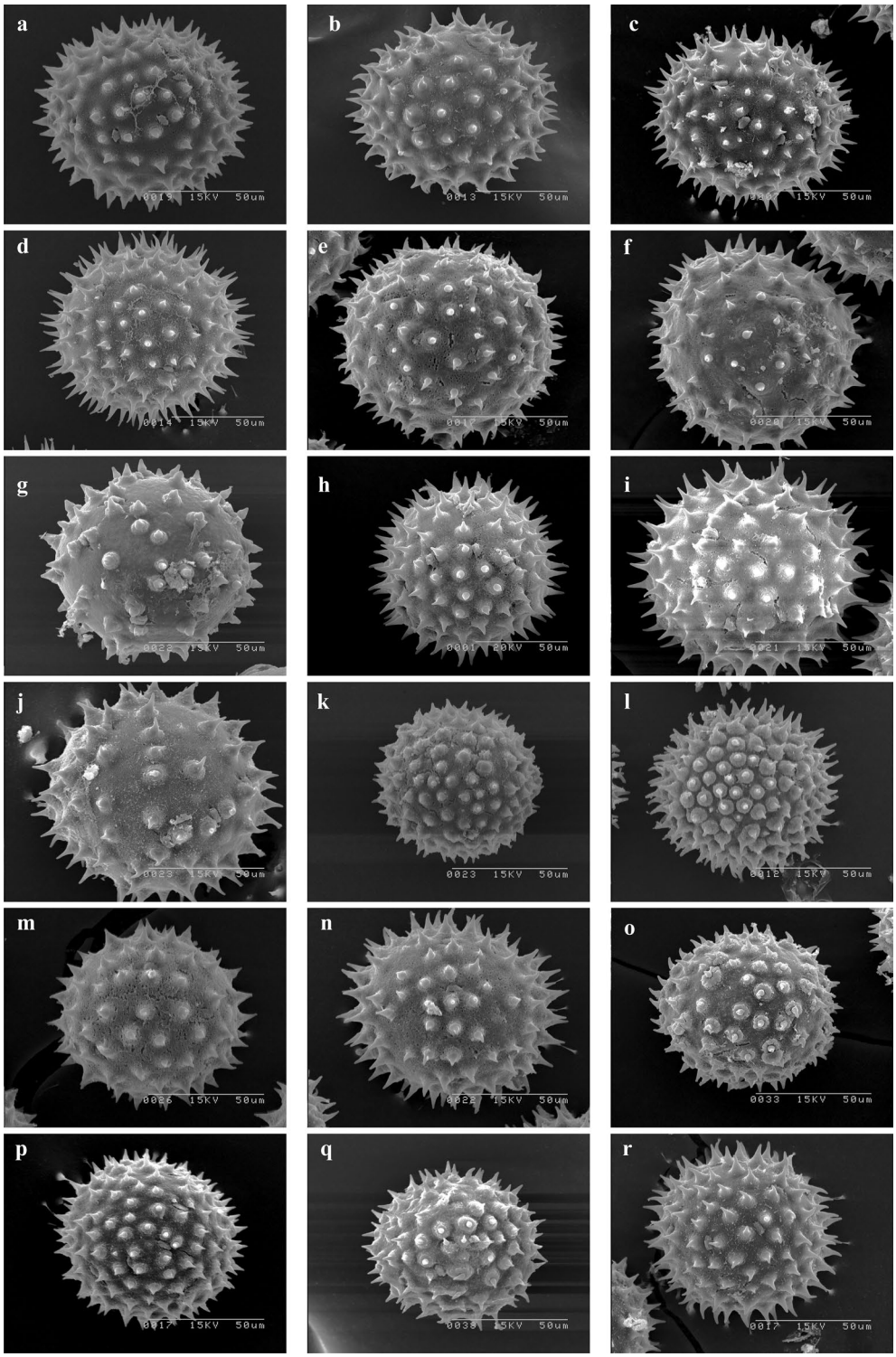

Fig. 1 SEM micrographs of pollen grains in *Gossypium*. **a** *G. hirsutum* (CRI12), **b** *G. barbadense* (CL), **c** *G. barbadense* (YM), **d** *G. barbadense* (XH7), **e** *G. tomentosum* (LZ), **f** *G. tomentosum* (JFZ8), **g** *G. tomentosum* (01), **h** *G. mustelinum* (LZ), **i** *G. mustelinum* (16), **j** *G. darwinii* (07), **k** *G. herbaceum* (ZC1), **l** *G. herbaceum* (africanum), **m** *G. arboreum* (SXY), **n** *G. arboreum* (Rozi), **o** *G. anomalum* (LZ), **p** *G. capitis-viridis* (LZ), **q** *G. capitis-viridis* (01), **r** *G. thurberi* (LZ), **s** *G. thurberi* (35), **t** *G. armourianum* (LZ) **u** *G. harknessii* (LZ), **v** *G. davidsonii* (LZ), **w** *G. klotzschianum* (LZ), **x** *G. raimondii* (01), **y** *G. gossypioide s*(LZ), **z** *G. schwendimani i*(LZ), **aa** *G. stocksii* (LZ), **ab** *G. somalense* (LZ), **ac** *G. areysianum* (LZ), **ad** *G. incanum* (04), **ae** *G. bickii* (LZ), **af** *G. australe* (LZ), **ag** *G. rotundifolium* (LZ). Scale bar: 50 μm. Accelerating voltage: 15 kV

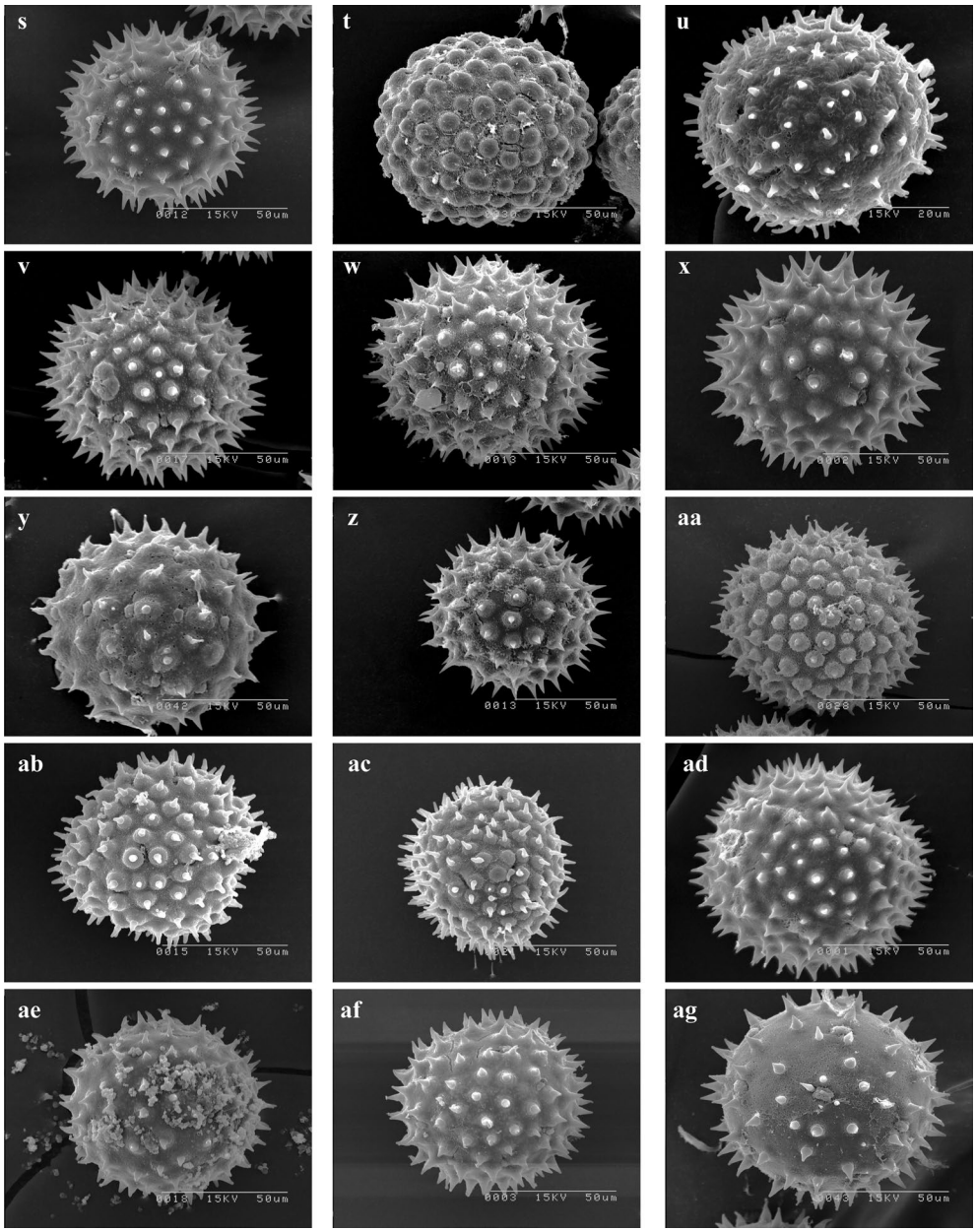

Fig. 1 continued

G. davidsonii, *G. raimondii*, *G. schwendimanii*, and *G. rotundifolium*). Cluster II contained 13 diploid species (*G. herbaceum*, *G. arboreum*, *G. anomalum*, *G. capitis-viridis*, *G. thurberi*, *G. armourianum*, *G. harknessii*, *G. gossypioides*, *G. stocksii*, *G. somalense*, *G. areysianum*, *G. bickii*, and *G. australe*). Cluster III comprised a single species (*G. incanum*).

According to the cluster analysis, the genus *Gossypium* was divided into three pollen types (Fig. 4). A key to pollen types and general pollen morphology of the genus *Gossypium* was provided in Table 2 (mean values were shown). Members of type-I pollen were characterized by the larger pollen size (almost all diameters > 80 μm), the longer exine spine (almost all lengths > 6 μm), exine spine numbers ranging from 57 to 117, the sharply conical exine spine, and the small or medium protuberance at the base of exine spine. Type-II members had the following features: the smaller pollen size (almost all diameters < 80 μm), the shorter exine spine (almost all lengths < 6 μm), exine spine

Table 2 Pollen morphologies of *Gossypium* species

Number	Material name	Pollen shape	Pollen diameter /μm	Echini length /μm	Echini density	Echini shape	Protuberance
1	*G.hirsutum* (CRI12)	Spheroidal	83.12–91.23 (87.07)	5.49–6.14 (5.78)	103–109 (105)	Sharply conical	Little
2	*G.barbadense*(CL)	Spheroidal	98.02–108.05 (103.41)	7.88–8.64 (8.29)	81–95 (89)	Sharply conical	Little
3	*G.barbadense*(YM)	Spheroidal	89.26–103.61 (96.09)	7.86–8.56 (8.20)	100–107 (104)	Sharply conical	Little
4	*G.barbadense*(XH7)	Spheroidal	98.82–110.68 (103.41)	9.07–10.00 (9.47)	110–123 (117)	Sharply conical	Little
5	*G.tomentosum* (LZ)	Spheroidal	94.86–104.29 (97.61)	5.53–5.96 (5.78)	74–80 (76)	Sharply conical	Little
6	*G.tomentosum*(JFZ8)	Spheroidal	86.30–98.88 (91.12)	6.17–6.98 (6.54)	60–70 (64)	Sharply conical	Little
7	*G.tomentosum*(01)	Spheroidal	87.67–100.66 (91.53)	5.51–5.88 (5.72)	52–62 (57)	Sharply conical	Medium
8	*G.mustelinum*(LZ)	Spheroidal	86.39–97.55 (92.49)	8.35–9.32 (8.81)	81–96 (89)	Sharply conical	Little
9	*G.mustelinum*(16)	Spheroidal	86.64-–94.96 (91.08)	7.59–8.44 (8.01)	80–91 (87)	Sharply conical	Little
10	*G.darwinii*(07)	Spheroidal	90.91–96.54 (93.28)	7.82-–8.45 (8.12)	63–72 (67)	Sharply conical	Medium
11	*G.herbaceum*(ZC1)	Spheroidal	65.93–73.47 (70.05)	3.30–3.91 (3.53)	102–114 (107)	Conical	Obvious
12	*G.herbaceum* (africanum)	Spheroidal	67.05–84.43 (73.41)	6.25–7.25 (6.86)	121–139 (128)	Conical	Obvious
13	*G.arboreum* (SXY)	Spheroidal	75.77–85.45 (80.36)	5.63–6.37 (5.97)	67–82 (74)	Conical	Little
14	*G.arboreum* (Rozi)	Spheroidal	75.63–87.17 (81.16)	7.19–7.97 (7.69)	90–98 (94)	Conical	Little
15	*G.anomalum* (LZ)	Spheroidal	69.63–76.22 (73.46)	4.76–5.67 (5.14)	73–81 (77)	Conical	Obvious
16	*G.capitis-viridis*(LZ)	Spheroidal	66.94-–70.47 (68.44)	4.55–4.88 (4.70)	78–90 (84)	Conical	Medium
17	*G.capitis-viridis*(01)	Spheroidal	68.42–75.14 (71.69)	5.22–5.45 (5.36)	77–90 (83)	Conical	Medium
18	*G.thurberi* (LZ)	Spheroidal	69.11–77.76 (73.93)	5.74–6.59 (6.19)	87–100 (93)	Conical	Little
19	*G.thurberi* (35)	Spheroidal	71.55–75.65 (74.02)	5.90–6.67 (6.35)	89–100 (94)	Conical	Little
20	*G.armourianum*(LZ)	Spheroidal	62.89–77.05 (69.73)	/	/	/	/
21	*G.harknessii* (LZ)	Spheroidal	58.55-–68.24 (62.43)	3.78–4.41 (4.07)	103–108 (106)	Rodlike	Little
22	*G.davidsonii* (LZ)	Spheroidal	77.31–82.15 (79.50)	6.01–7.36 (6.78)	97–116 (103)	Sharply conical	Little
23	*G.klotzschianum* (LZ)	Spheroidal	85.09–90.42 (87.22)	5.51–6.17 (5.90)	108–118 (113)	Sharply conical	Little
24	*G.raimondii*(01)	Spheroidal	85.67–89.11 (86.71)	6.40–7.39 (6.83)	79–93 (86)	Sharply conical	Little
25	*G.gossypioides*(LZ)	Spheroidal	75.65–81.40 (77.99)	5.35–5.96 (5.66)	50–60 (54)	Conical	Medium
26	*G.schwendimanii*(LZ)	Spheroidal	82.03–86.17 (84.16)	7.85–8.25 (8.09)	62–72 (67)	Sharply conical	Medium
27	*G.stocksii* (LZ)	Spheroidal	77.26–86.08 (81.98)	4.86–5.98 (5.34)	117–128 (121)	Conical	Obvious
28	*G.somalense*(LZ)	Spheroidal	72.40–82.97 (79.14)	4.95–5.63 (5.36)	94–100 (96)	Rodlike	Obvious
29	*G.areysianum* (LZ)	Spheroidal	66.26–74.39 (69.80)	5.23–6.09 (5.68)	106–119 (112)	Rodlike	Obvious
30	*G.incanum* (04)	Spheroidal	67.28–77.92 (72.00)	3.70–4.68 (4.21)	165–178 (173)	Conical	Little
31	*G.bickii* (LZ)	Spheroidal	63.08–76.30 (69.78)	4.91–5.87 (5.31)	75–87 (79)	Conical	Little
32	*G.australe* (LZ)	Spheroidal	69.98–64.66 (64.66)	5.65–6.09 (5.88)	85–93 (90)	Conical	Little
33	*G.rotundifolium* (LZ)	Spheroidal	83.29–92.25 (87.25)	7.05–7.93 (7.39)	69–78 (75)	Sharply conical	Little

The datums in brackets represent the average values; /, the data is missing

numbers ranging from 54 to 128, any of the three exine spine shapes, and the medium or obvious protuberance at the base of the exine spine. Finally, pollen characteristics in species of type-III were the smaller pollen size (diameter = 72 μm), the shorter exine spine (4.21 μm), the high exine spine density (173), and the small protuberance at the base of the exine spine.

Discussion

Various species of *Gossypium*, although distinct morphologically, have many shared features. Pollen grains of all species examined in this study are monad, radially symmetrical, spherical, echinate, panporate, and operculate. These results are consistent with previous studies (Jia et al. 1988; Lan and Xu 1996; Saensouk and Saensouk 2021). For example, Saensouk and Saensouk (2021) has investigated the pollen morphology of subfamily Malvoideae in Thailand and has found that pollen grains of all 19 species (including *G. barbadense*) are monad, spheroidal, panporate, apolar and radially symmetrical. Pollen shape, which is based on the ratio of polar diameter (P) to equatorial diameter (E), can be classified into five categories, i.e., oblate (P/E < 0.50), suboblate (P/E = 0.5–0.88), spheroidal (P/E = 0.88–1.14), subprolate (P/E = 1.14–2.00), and prolate (P/E > 2.00) (Erdtman 1987). In this study, all investigated species have a spheroidal pollen shape, which is congruent with the results of previous studies (Jia et al. 1988; Lan and Xu 1996). Significant

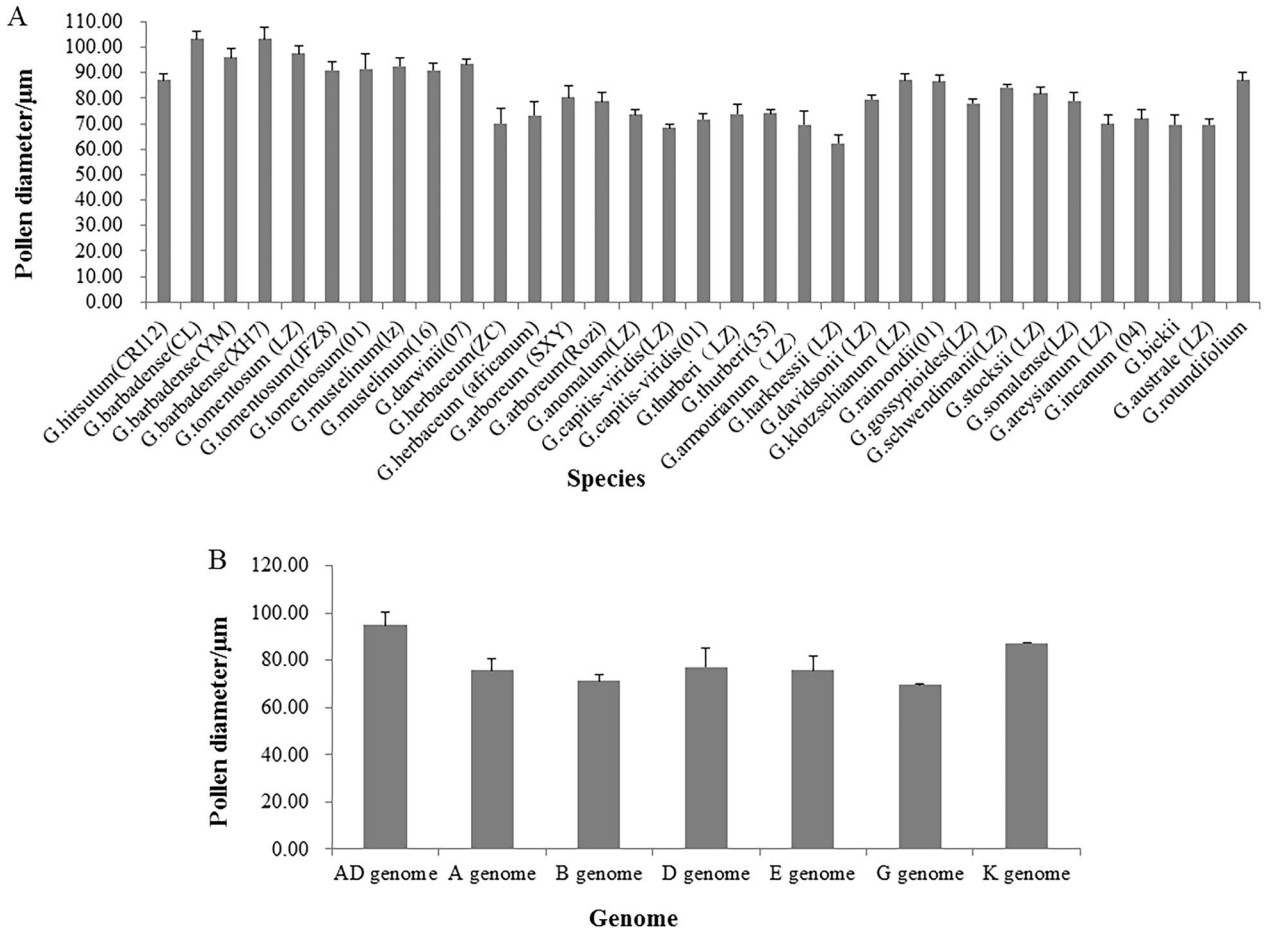

Fig. 2 The diameter of pollen in the *Gossypium* species. **A** Comparison of pollen diameter among *Gossypium* species. **B** Comparison of pollen diameter among different genomes of *Gossypium*

Table 3 The Pearson correlation test between genome size and pollen size in *Gossypium* genus

		Genome size	Pollen size
Genome Size	Pearson correlation coefficient (*r*)	1	0.648**
	Significance (*p*)		0.000
	Sample number (*N*)	33	33
Pollen Size	Pearson correlation coefficient	.648**	1
	Significance (*pP*)	.000	
	Sample number (*N*)	33	33

**Represents the correlation reached extremely significant ($p < 0.01$)

variations in pollen size and sculpture are potentially very useful for species identification and delimitation (Ullah et al. 2018). In this study, we have observed considerable variation in pollen size between different cotton species, but little variation within one species. This pattern suggests that pollen size is a useful taxonomic parameter at the species level.

As is well known, pollen size usually increases with chromosome number. For instance, the pollen grain sizes of tetraploid species of the genus *Skimmia* tend to be larger compared with those of diploid species, but this is not always true (Fukuda et al. 2008). In the present study, we also observed that the pollen size of allotetraploids ($4n = 52$) had larger pollen sizes relative to diploid cotton species ($2n = 26$). The most widespread consequence of polyploidy in plants is resulting increased cell size due to the larger number of gene copies. Consequently, polyploid individuals may exhibit larger organs, such as roots, leaves, tubercles, fruits, flowers, and seeds, compared with the diploid (Sattler et al. 2016). Interestingly, we also observed that pollen of the diploid K-genome species (*G. rotundifolium*) are larger than other diploid cotton species. The increased pollen size of *G. rotundifolium* may due to its larger genome, evenif the average genome size of K-genome species (~2 570 Mb) is only slightly larger

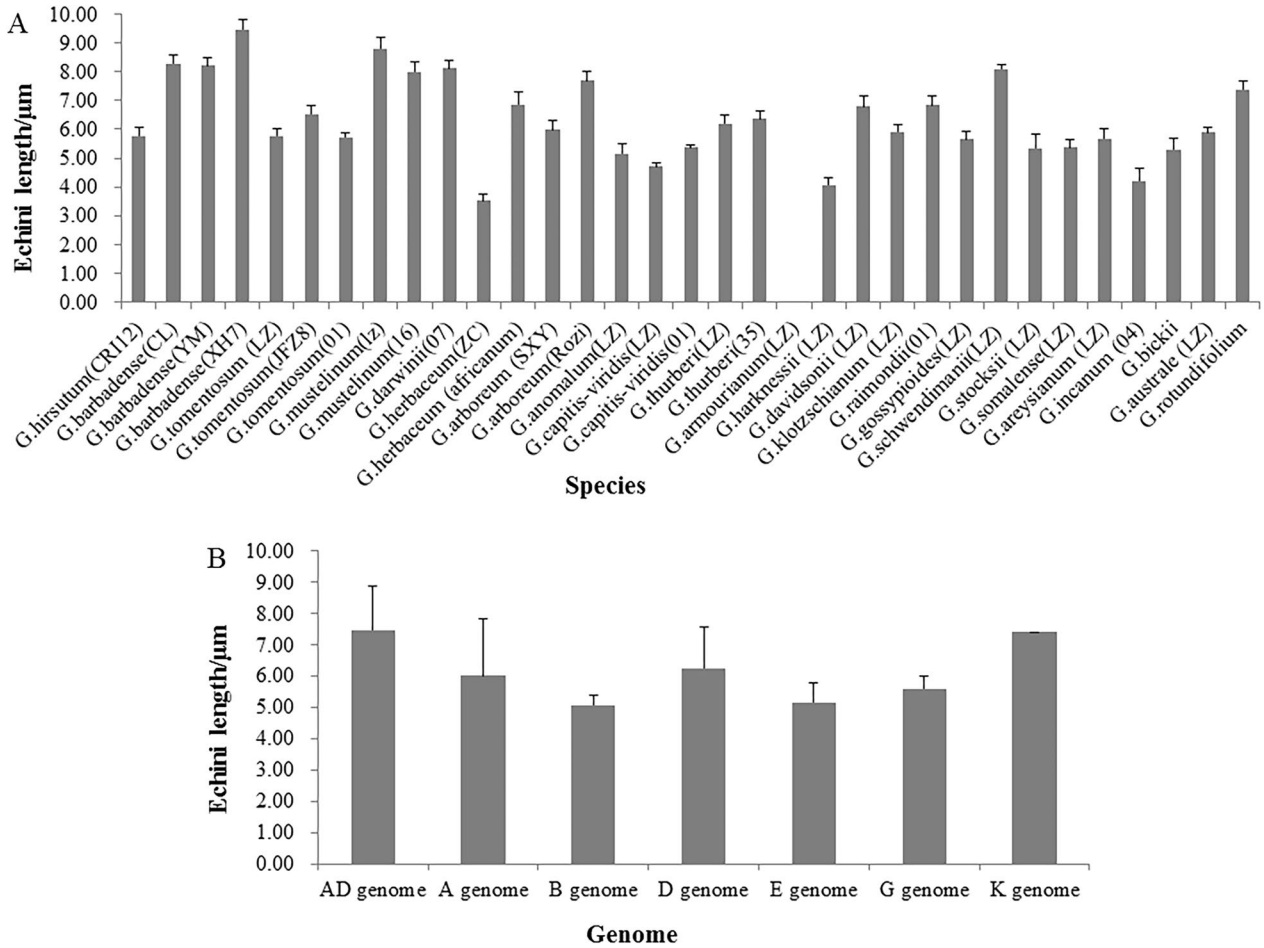

Fig. 3 The length of exine spine in the *Gossypium* species. **A** Comparison of exine spine length among *Gossypium* species. **B** Comparison of exine spine length among different genomes of *Gossypium*

Table 4 The Pearson correlation test between pollen diameter and spine length in *Gossypium* genus

		Pollen diameter	Spine length
Pollen diameter	Pearson's correlation coefficient (*r*)	1	0.690**
	Significance (*p*)		0.000
	Sample number (*N*)	33	33
Spine length	Pearson correlation coefficient	.690**	1
	Significance (*P*)	.000	
	Sample number (N)	33	33

**Represents the correlation reached very significant (P < 0.01)

than that of AD-genome allotetraploids (~2 400 Mb) (Wendel et al. 2012). In the present study, Pearson correlation analysis uncovered a significant positive correlation ($r = 0.65$, $P < 0.01$) between genome size and pollen size in cotton.

As noted in previous studies, the exine ornamentation of pollen is an important diagnostic character for delimiting relationships within tribes, families, and genera in Brassicaceae (Khalik et al. 2002; Erik 2012). In addition, Baser et al. (2016) have reported that exine ornamentation is useful for distinguishing between closely related species in the same genus, such as *Pelargonium endlicherianum* and *P. quercetorum*. In the present investigation, we have found that the pollen exine of

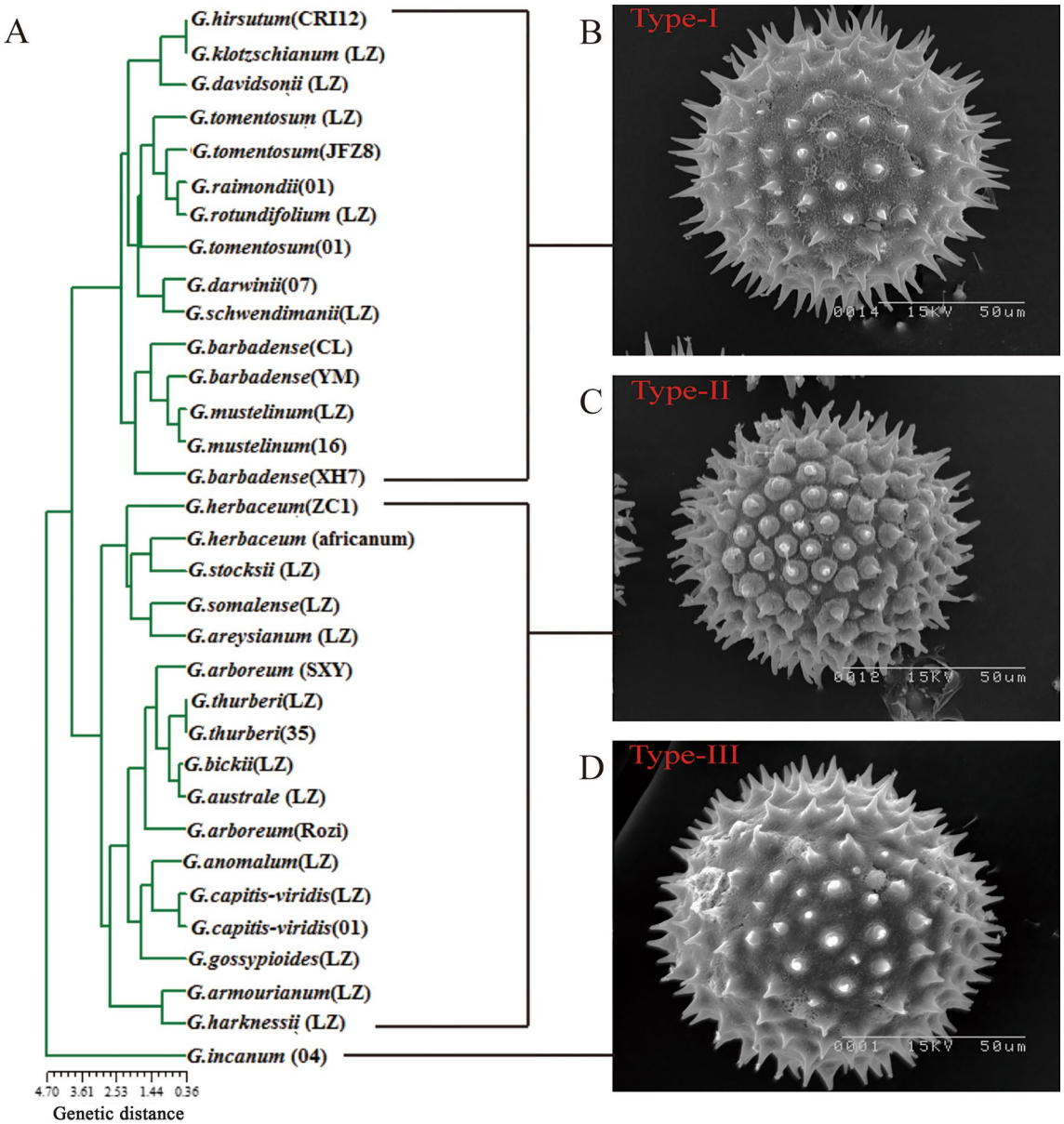

Fig. 4 Dendrogram of *Gossypium* based on pollen characteristics (**A**). Three pollen types (right): Type I, big pollen size, long spine length, low spine density and little protuberance (**B**); Type II, small pollen size, short spine length, low spine density, and obvious protuberance (**C**); Type III, small pollen size, short spine length, high spine density, and little protuberance (**D**). Scale bar: 50 μm. Accelerating voltage: 15 kV

all cotton species was densely covered with spines with one exception: *G. armourianum,* whose pollen surface is covered with a thick layer of wax. On the basis of shape, exine spines of cotton pollen grains are separated in two main types: conical or rod-like. The most common exine spine shape in *Gossypium* is conical (19 species), and a rod-like shape only found in three species. Judging from the SEM photographs, the base of the exine spine commonly bulges to form a tuberculate structure (called as protuberance), but the size of the protuberance varies among the investigated cotton species. Although pollen grains of *Gossypium* species usually have echinate exine ornamentation, most of them have different spine densities and exine spine lengths. In addition, the results of Pearson correlation analysis indicate that cotton pollen size is highly correlated with exine spine length ($r = 0.69$, $P < 0.001$) while weakly correlated with exine spine number ($r = -0.04$, $P > 0.05$). This suggests that cotton species with larger pollen sizes have longer exine spines. For example, the diploid cotton species *G. schwendimanii*

has larger pollen grains and longer exine spines but fewest number of exine spines. On the contrary, *G. incanum* has smaller pollen grains and shorter exine spines but with the most exine spines. In regard of *G. armourianum*, the thick wax coating on its pollen grains is difficult to remove, we are unable to observe and analyze the exine spine during multiple preliminary experiments using pollen from materials of different years. Nevertheless, we believe that *G. armourianum* has exine spines, as the presence of echinate sculpture is a conserved feature in the Malvaceae family. And *G. harknessii*, the sister species of *G. armourianum* has normal exine spines. Further study, however, is necessary to fully reveal the pollen characteristics of *G. armourianum*.

Cluster analysis based on the six studied pollen traits has grouped all allotetraploid species (AD genome group) into Cluster I, with the wild cotton *G. tomentosum* (AD_3) showing a closer relationship to the cultivated cotton *G. hirsutum* (AD_1) compared with other allotetraploid species. Some placements were confusing and unexpected, for example, the nine cotton species from the D-genome group are distributed on different cluster branches, and *G. incanum* (E_4) is separated from other E-genome species (E_1, E_2, and E_3). Although the cluster dendrogram was not exactly coincident with the molecular phylogenetic tree proposed by Wendel and Cronn (2003), the results of this analysis provide new information, from a palynological perspective, on phylogenetic relationships among 23 analyzed *Gossypium* species. A sole reliance on pollen characteristics is thus obviously insufficient for understanding taxonomic and evolutionary relationships within the genus *Gossypium*. Nevertheless, pollen morphological variations are of significance for further taxonomic classification at the species level.

Conclusions

In this study, we acquired SEM images and pollen morphological data for the largest number of cotton species reported to date. We found that pollen size, ornamentation and spine features among species are distinctive and can be used to understand the interspecific relationship. The morphological description from this study not only expands the knowledge in cotton palynomorphology but also sheds light on the palynomorphology based phylogenetic analysis among *Gossypium* species.

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Author contributions
Cai XY performed the experimental procedures and drafted the manuscript. Hou YQ and Wang H analyzed the data. Muhammad JU revised the language. Xu YC, Zheng J, and Wang YH carried out material management. Liu F, Zhou ZL, and Hua JP revised the manuscript. Wang KB designed the study. All authors read and approved the final manuscript.

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Availability of data and materials
The related genome size data of all cotton were collected and downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). All data generated or analyzed during this study are included in this article. The datasets and original figures used and analyzed in this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
All the cotton germplasm resources used in this research were preserved in the National Wild Cotton Germplasm Resources Nursery (Sanya, China). Experimental research on plants in this study complied with institutional, national, or international guidelines and legislation. All the experiments were performed in accordance with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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