RESEARCH ARTICLE

Pollen Morphological Variations in Hybrids of *Hibiscus rosa-sinensis* L.: Implications for Palyno-taxonomy

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Abstract

Human-induced cross-pollination is widely done in *Hibiscus rosa-sinensis* L. (family Malvaceae), an ornamental perennial plant, for its beautiful flower colors. Pollen morphometrical study of 15 hybrids were studied using light microscopy (LM), confocal laser scanning microscopy (CLSM) and field emission scanning electron microscopy (FESEM) to document the ultrastructure of pollen and to facilitate palynological studies. The shape of all the grains is radially symmetrical, sub-spheroidal and apolar measuring from 35 to 70 µm in size. However, variations in pollen shape from spheroidal, prolate spheroidal and sub-prolate have been observed. The aperture in all the specimens is pantoporate which is typical of its family. The pollen surface is echinate (grains having spines) in all the hybrids. The details of exine ornamentation, spine morphological variations and size using FESEM and CLSM majorly show high variability in all the 15 hybrids. The exine thickness varied between 1 to 4 µm with length varying from 5 to 10 µm. The bifurcated and branched echinae, spinules were observed in *Hibiscus* hybrids. The pollen size significantly correlates with the length of the spine and the color of the flower obtained after cross-pollination. The principal component analysis (PCA) shows significant variability in polar axis/polar length, equatorial diameter, pore length, pore width, spine length, spine width and spine base, which identifies morphological characters within the hybrids. We infer that size, shape, and spine length in pollen distinguishes *Hibiscus* and its hybrids from other genus in the family. All the studied hybrids show high variability in spine index and branching pattern. The study provides information to strengthen taxonomic and palynological studies and in understanding the pollen morphometrical variability in natural and man-made plant hybrids.

Keywords: *Hibiscus rosa-sinensis* L. pollen; hybrids; spine index; taxonomy and systematics; India

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Introduction

Large ecological differences exist between species within genera and between genera within plant families. In a changing environment, heterogamy (cross-pollination) is beneficial for plants as hybrid plants have a mixture of genetic characteristics and tend to be more successful in adjusting to changing environmental conditions, which started millions of years back with the evolution of angiosperms, in particular. This process still continues in nature, but humans have used scientific advances and new techniques for long to produce hybrid plants for various reasons. Hence, the pollen morphological variations are apparent and many times, it becomes difficult to assign a defined genus. Therefore, low taxonomic resolution constrains the paleoecological and paleoenvironmental inferences that can be drawn from fossil pollen analysis, which remains a key obstacle in both Quaternary and pre-Quaternary palynology (Birks and Birks, 2000; Jackson and Booth, 2007; Mander, 2011; Punyasena *et al*., 2011, 2012, May and Lacourse, 2012; Lacourse and May, 2012; Mander *et al.,* 2013; Muller, 1981; Tellerı́a *et al.,* 2010). Long-term evolution that takes place through a geological time period develops a variety of pollen morphology, which are controlled by genes and are least affected by the external natural environment. Hence, pollen morphology has an important role in plant taxonomy, evolution and identification. The cross-pollination has often been used to produce more and better quality of seeds, increasing the survival rate and productivity. This technology is more important to improve certain crops and ornamental plants.

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The variability in the pollen micro-morphology of several natural and man-made plant hybrids has been frequently observed in terms of the number of pollen apertures, size, shape and exine ornamentation (KarlsdóTtir *et al.,* 2008). In the modern world, in order to increase the aesthetic and ornamental values, *Hibiscus rosa-sinensis* L. has been used by human beings to produce different shades of color in flowers by cross-pollination technique, which is commonly done manually. To create different colors in hybrid plants, the pollen is exchanged between two varieties of the same plant. The most frequent anomaly observed in the pollen morphology of a hybrid plant is its variability from their respective taxonomic characters known for a parent genus. Palynological studies have been frequently used in the taxonomy and phylogeny of several taxa (Ferguson, 1986; Hilsebeck, 1990; El Naggar, 2002). Pollen morphology of Malvaceae or some of its representatives has been included in many studies: for example, Surova and Velieve (1984), Christensen (1986a, 1986b), Culhame and Blackmore (1988) and El Naggar (2004). The pollen of Malvaceae is characterized by large size, spherical shape, corporate or pastorate aperture and echinate sculpture. Apart from these characteristics, they vary greatly in hybrids, which can be used in the classification and phylogeny within the genus and family (Christensen, 1986 a, 1986b; Culhame and Blackmore, 1988; El Naggar, 2004).

Increasing the taxonomic precision of modern (hybrid) and fossil pollen identification (for the reconstruction of past vegetation dynamics and contemporary climate change) relies directly on the study of pollen morphology. The foundation of palaeoecological reconstructions (of vegetation dynamics) is, in fact, accurate and precise fossil pollen identification, which is based on the correlation between modern pollen morphology and fossil pollen found in sedimentary archives. It is based on the assumption that pollen morphology has not changed through time, with a few exceptions (Lacourse and May 2012). Pollen of *Hibiscus rosa-sinensis* L. is characterized by spheroidal-sub spheroidal echinate ornamentation that displays a 'fullerene' structure of Carbon bonds concerning the distance between the spines. However, natural and man-made hybrids of plants produce pollen which tend to deviate in morphology and can be well studied using high-resolution microscopic techniques such as FESEM and CLSM. This study aims to document the pollen ultrastructure of the *Hibiscus rosa-sinensis* L. hybrids and to differentiate the pollen traits via numerical analysis to evaluate the pollen morphometry of hybrids and its taxonomic significance.

Materials and Methods

Pollen samples of 15 hybrids of *Hibiscus rosa-sinensis* L. plant (including the species in question) were obtained from the plants growing on the premises of the Birbal Sahni Institute of Palaeosciences (BSIP, Lucknow), The Citadel Apartments (Chinhat, Lucknow) and the Vivekanand Matth near Vivekanand Hospital at Lucknow, Uttar Pradesh, India (Fig.1; Table1). All the selected taxa are the hybrids of *Hibiscus rosa-sinensis* L. The terminology used for describing the pollen morphology of the hybrids of *Hibiscus rosa-sinensis* L. is based on Erdtman (1952), Faegri and Iversen (1964, 1975), Walker and Doyle (1975), Punt *et al*. (2007) and Hesse *et al*., (2009).

Fig. 2. A. Flower of *H. rosa sinensis* L. H, B. LM microphotograph; B: exine, pores and spines focussed, C. CLSM image: C. Spines, pores and surface features focussed; D. FESEM image; D. spines focussed along with surface views, complete grain seen; E. Flower of *H. rosa sinensis* L. H1, F. LM microphotograph; F: exine, pores and spines focussed, G. CLSM image: G. exine, spines and pores focussed along with surface features, H. FESEM image; H. spines focussed; surface views seen; I. Flower of *H. rosa sinensis* L. H2, J. LM microphotograph; J. spines and pores focussed, along with the exine, K. CLSM image: K. pores, exine, spines focussed, and surface features seen, L. FESEM image; L. spines focussed and complete grain seen with surface views, showing circular depressions on the surface, M. Flower of *H. rosa sinensis* L. H3, N. LM microphotograph; n. spines focussed, clearly showing bulbous spine bases, along with and pores, O. CLSM image: O. pores, exine, spines focussed, and surface features seen, P. FESEM image; P. spines focussed and surface views seen with techniques, such as perforations and minute granules, Q. Flower of *H. rosa sinensis* L. H4, R. LM microphotograph; R. exine, and pores focussed along with spines, S. CLSM image: S. surface view seen along with spines, exine and pores, T. FESEM image; T. spines focussed in a complete grain.

Fig. 3. Flower of *H. rosa sinensis* L. H5, B. LM microphotograph; B. exine focussed, a few pores are also visible, impressions of spines seen at the exine surfaces, surface views also seen, C. CLSM image: C. spines and pores focussed along with exine, as well as surface features, D. FESEM image; D. only impressions of spines and surface features seen, E. Flower of *H. rosa sinensis* L. H6, F. LM microphotograph; F. spines focussed showing distinct spines bases, pores also seen, G. CLSM image: G. spines, and pores focus, along with surface features,H. FESEM image; H. spines focussed along with surface views showing depressions on the surface with minute granules in a complete grain, I. Flower of *H. rosa sinensis* L. H7, J. LM microphotograph; J. spines, pores and exine focussed, K. CLSM image: K. spines focussed, pores, exine and surface features also seen, L. FESEM image; L. spines focussed along with surface features showing bifurcations, surface features seen along with perforations on the surface, M. Flower of *H. rosa sinensis* L. H8, N. LM microphotograph; N. exine, spines and pores focussed, O. CLSM image: O. pores, exine, spines focussed, surface features also seen, P. FESEM image; P. spines focussed along with surface view in a complete grain, pores also visible along with depressions and minute granules, Q. Flower of *H. rosa sinensis* L. H9, R. LM microphotograph; R. spines and pores focussed, along with exine, S. CLSM image: S. Spines, pores, and exine focussed, surface features also seen along with a few spines, T. FESEM image; T. spines focussed in a complete grain, spines also showing bifurcations, surface views also seen.

Fig. 4. Flower of *H. rosa sinensis* L. H10, B. LM microphotograph; B. spines and exine focussed, a few pores also evident along with impressions of some other pores, a surface view also seen, C. CLSM image: C. spines and pores, focussed, surface features also seen, D. FESEM image; D. spines focussed along with surface features, showing perforations and minute granules, E. Flower of *H. rosa sinensis* L. H11, F. LM microphotograph; F. spines focussed showing distinct spine bases and apices, G. CLSM image: G. spines focussed showing a few pores too along with surface features, H. FESEM image; H. spines focussed in a complete grain, also showing surface views and minute granules, I. Flower of *H. rosa sinensis* L. H12, J. LM microphotograph; J. exine focussed showing a few spines and pores, surface features also seen, K. CLSM image: K. spines and pores focussed along with exine, spines also show bifurcations, L. FESEM image; L. spines focussed in a complete grain, surface features also seen, spines showing bifurcations also visible, along with depressions on the surface of the grain, minute granules on the surface seen, M. Flower of *H. rosa sinensis* L. H13, N. LM microphotograph; n. spines focussed showing distinct spine apices and bases, along with surface features, O. CLSM image: O. spines and pores focussed, surface features also seen, P. FESEM image; p. spines focussed in a complete grain, showing distinct surface features having perforations and granules, Q. Flower of *H. rosa sinensis*

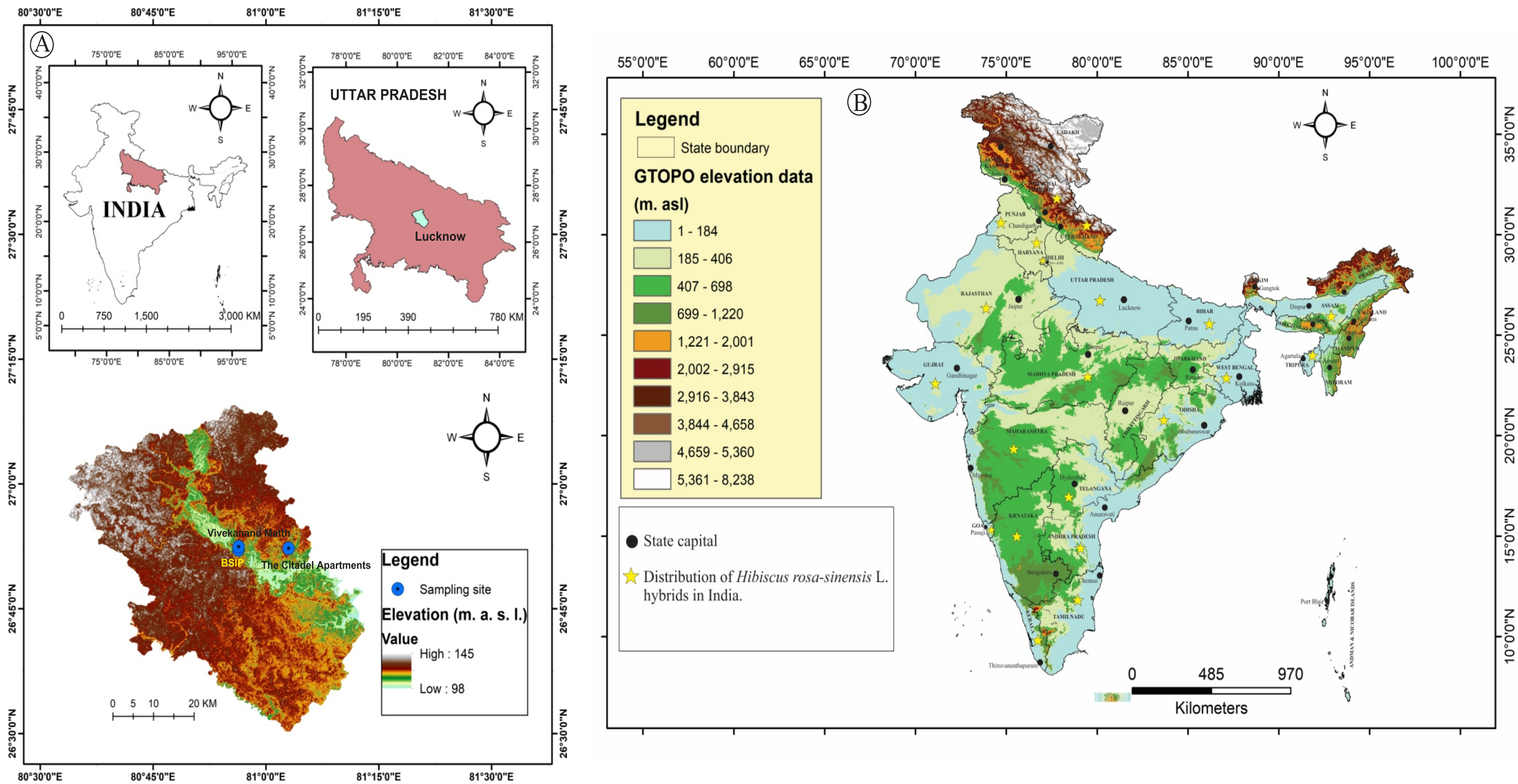

Fig. 1: 1A. Shuttle radar topographic mission (SRTM) digital elevation model (DEM) of the Lucknow District (Uttar Pradesh), India, showing the location (BSIP, Vivekanand Matth, and The Citadel Apartments: Chinhat, Lucknow) of the study area ("blue circles" shows the sampling site: BSIP, Vivekanand Matth and The Citadel Apartments: Chinhat, Lucknow), geographical map of India showing the State: Uttar Pradesh (inset right), geographical map of Uttar Pradesh State showing the Lucknow District (inset left). Source of Figure. 1A: The Figure 1A has been made using ArcGIS 10.3.**, 1B.** GTOPO 30 digital elevation model (DEM) of India, showing the distribution of the 15 studied *Hibiscus rosa-sinensis* L. hybrids in the Indian subcontinent. Source of Figure 1B: Figure 1B has been made using ArcGIS 10.3 (Global 30 Arc-Second Elevation (GTOPO30) Digital Object Identifier (DOI) number: /10.5066/F7DF6PQS).

Table 1: Salient pollen morphological attributes (quantitative and qualitative measurements) of the 15 studied *Hibiscus rosa-sinensis* L. hybrids (Malvaceae), as well as their respective reference laboratory numbers (of living pollen slides).

S. No.	*Plant taxa*	*Size*	*(P/E) (μm)*	*Shape*	*Aperture (no., nature and diameter)*	*Pollen wall (exine) architecture, diameter and base of echina and its dispensation*
1.	*Hibiscus rosa- sinensis* L. (HRS) Flower red color with yellow shade. RLN: 11805 (BSIP)	VL	50/50	SS/OS	Pantoporate, pore (2 × 2 μm); variable	exine 2 μm thick, echina: length 6 μm, width 2 μm, base 3 μm, spine index 6/2 μm, spines are dimorphic and have rounded apices with slightly swollen bases, pattern psilate; however, surface seems to be rough having number of ridges upon which spines are located (in FESEM images)
2.	*Hibiscus rosa- sinensis* L. (HRS H1) Flower pure yellow and dark yellow color at the base. RLN: 11806 (BSIP)	VL	60/60	SS/OS	Pantoporate, pore (2 × 2 μm), variable	exine 2 μm thick, echina: length 9 μm, width 2 μm, base 4 μm, spine index 9/2 μm, spines are dimorphic and have, although, rounded apices, but sometimes curved and directed towards the periphery, have slightly swollen bases and bifurcation too, pattern mostly obscure (psilate); but sometimes rough surface is seen (in FESEM images)
3.	*Hibiscus rosa- sinensis* L. (HRS H2) Flower pure white. RLN: 11907 (BSIP)	VL	60/60	SS/OS	Pantoporate, pore (2 × 2 μm); variable	exine 3 μm thick, echina: length 9 μm, width 2 μm, base 3 μm, spine index 9/2 μm, spines are dimorphic, showing both acute and rounded apices and slightly swollen bases, some spines are directed away from the centre and are curved as well, pattern psilate; however, rough surface with depression is also seen (in FESEM images)
4.	*Hibiscus rosa- sinensis* L. (HRS H3) Flower dark pink-shaded. RLN: 11808 (BSIP)	VL	70/70	SS/OS	Pantoporate, pore (2 × 2 μm); variable	exine 1.5 μm thick, echina: length 6 μm, width 1.5 μm, base 2 μm, spine index 6/1.5 μm, spines are dimorphic with rounded apices and slightly swollen bases, pattern psilate; however, rough surface with minute granules are also seen (in FESEM images)
5.	*Hibiscus rosa- sinensis* L. (HRS H4) Pink flower with white shade. RLN: 11809 (BSIP)	L	65/68	SS/OS	Pantoporate, pore (2 × 2 μm); variable	exine 4 μm thick, echina: length 7 μm, width 1 μm, base 2 μm, spine index 7/1 μm, spines are dimorphic, having both rounded, as well as blunt and acute apices, especially of those exines which are of bifurcated ones; multiple bifurcations are seen even in a single spine, the bases of spine are slightly swollen, pattern psilate, but surface with varied ridges (circular, elongated, equatorially elongated, etc.) with minute granules are seen (in FESEM images)
6.	*Hibiscus rosa- sinensis* L. (HRS H5) Flower white creamy double and dark red color at the base. RLN: 11810 (BSIP)	VL	45/41	SS/OS	Pantoporate, pore (2 × 2 μm); variable	exine 2.5 μm thick, echina: length 5 μm, width 1 μm, base 3 μm, spine index 5/1 μm, spines are dimorphic having rounded apices and slightly swollen bases, pattern psilate; however, very rough surface is seen (in FESEM images)
7.	*Hibiscus rosa- sinensis* L. (HRS H6) Flower light peach in color. RLN: 11811 (BSIP)	VL	58/56	SS/PS	Pantoporate, pore (2 × 2 μm); variable	exine 3 μm thick, echina: length 10 μm, width 3 μm, base 4 μm, spine index 10/3 μm, spines are dimorphic having rounded apices and slightly bulbous bases, pattern obscure (psilate); however, rough surface with perforations and minute granules are also seen (in FESEM images)
8.	*Hibiscus rosa- sinensis* L. (HRS H7) Flower scarlet-red double flowers with ruffled petals. RLN: 11812 (BSIP)	L	55/60	SS/OS	Pantoporate, pore (2 × 1 μm); variable	exine 2 μm thick, echina: length 7 μm, width 3 μm, base 5 μm, spine index 7/3 μm, spines are mostly monomorphic with rounded apices and bulbous bases, huge bifurcations in the spines are evident with varied shapes and dispensation, such as curved spines with blunt and cute apices and slightly bulbous bases (directed towards the periphery), normal spines with rounded apices and bulbous bases are seen together, pattern psilate; however, rough surface with perforations are evident (in FESEM images)

9.	*Hibiscus rosa- sinensis* L. (HRS H8) Purple flower and light pink color at the base. RLN: 11813 (BSIP)	VL	50/50	SS/OS	Pantoporate, pore (2 × 2 µm); variable	exine 2 µm thick, echina: length 5 µm, width 1 µm, base 2 µm, spine index 5/1 µm, spines are dimorphic with both rounded and acute apices, showing some sort of curvedness towards the periphery, spine bases are slightly bulbous, pattern psilate, but rough surface with rounded and elliptic perforations are seen (in FESEM images)
10.	*Hibiscus rosa- sinensis* L. (HRS H9) Flower dark red in color. RLN: 11814 (BSIP)	VL	50/50	SS/OS	Pantoporate, pore (2 × 2 µm); variable	exine 2 µm thick, echina: length 9 µm, width 1 µm, base 1.5 µm, spine index 9/1 µm, spines are dimorphic having rounded apices and bulbous bases, spines show bifurcations with blunt and acute apices and slightly bulbous bases, pattern psilate; however, circular perforations are seen on the smooth surface (in FESEM images)
11.	*Hibiscus rosa-sinensis* L. (HRS H10) Flower golden yellow and white color at the base. RLN: 11815 (BSIP)	VL	35/33	SS/PS	Pantoporate, pore (2 × 2 µm); variable	exine 2 µm thick, echina: length 3 µm, width 1 µm, base 2 µm, spine index 3/1 µm, spines are dimorphic having both rounded and acute and blunt apices, spine bases swollen, minute bifurcations in the spine(s) are seen and sometimes show curvedness in that, directed towards the periphery, pattern psilate; however, perforations and some sort of roughness are also evident (in FESEM images)
12.	*Hibiscus rosa- sinensis* L. (HRS H11) Flower light pink in color and red color at the base. RLN: 11816 (BSIP)	VL	40/35	SS/SP	Pantoporate, pore (1 × 1 µm); varaible	exine 2 µm thick, echina: length 5µm, width 2 µm, base 3 µm, spine index 5/2 µm, spines are dimorphic having rounded apices and bulbous bases, bifurcations in spines are evident, pattern psilate; however, rough surface with rounded (mostly) perforations are seen (in FESEM images)
13.	*Hibiscus rosa- sinensis* L. (HRS H12) Flower pink with dark pink shade at the base. RLN: 11817 (BSIP)	VL	45/38	SS/SP	Pantoporate, pore (1 × 1 µm); variable	exine 1 µm thick, echina: length 5 µm, width 1 µm, base 2 µm, spine index 5/1 µm, spines are dimorphic having rounded, blunt and acute apices, bases of spines are slightly swollen, bifurcations in some spines are evident, which are directed towards the periphery, and pattern psilate; however, rough surface with minute granules, as well as varied perforations are clearly visible (in FESEM images)
14.	*Hibiscus rosa-sinensis* L (HRS H13) Flower dark yellow in color and white and dark red color at the base. RLN: 11818 (BSIP)	VL	65/55	SS/SP	Pantoporate, pore (2× 2 µm); varaible	exine 2 µm thick, echina: length 5 µm, width 2 µm, base 3 µm, spine index 5/2 µm, spines are monomorphic with rounded apices and bulbous bases, pattern psilate; however, rough surface with circular perforations are seen (in FESEM images)
15.	*Hibiscus rosa- sinensis* L. (HRS H14) Flower deep pink in color and dark yellow color at the base. RLN: 11819 (BSIP)	VL	60/60	SS/OS	Pantoporate, pore (2 × 2 µm); variable	exine 2 µm thick, echina: length 7 µm, width 2 µm, base 3 µm, spine index 7/2 µm, spines are dimorphic having rounded apices and bulbous bases, pattern psilate; however, circular perforations on the rough surface is evident (in FESEM images)

Abbreviations used in Table 1:
HRS= *Hibiscus rosa- sinensis* L., HRS H1-14: Hybrids of *Hibiscus rosa- sinensis* L.
L= large, VL = very large (Walker and Doyle, 1975), P/E = ratio of the measurements of the polar and equatorial axes.
SS = sub-spheroidal, OS = oblate spheroidal, PS = prolate spheroidal, SP = sub-prolate (Erdtman, 1954).
RLN = Reference laboratory number
BSIP= Birbal Sahni Institute of Palaeosciences, Lucknow (Uttar Pradesh), India

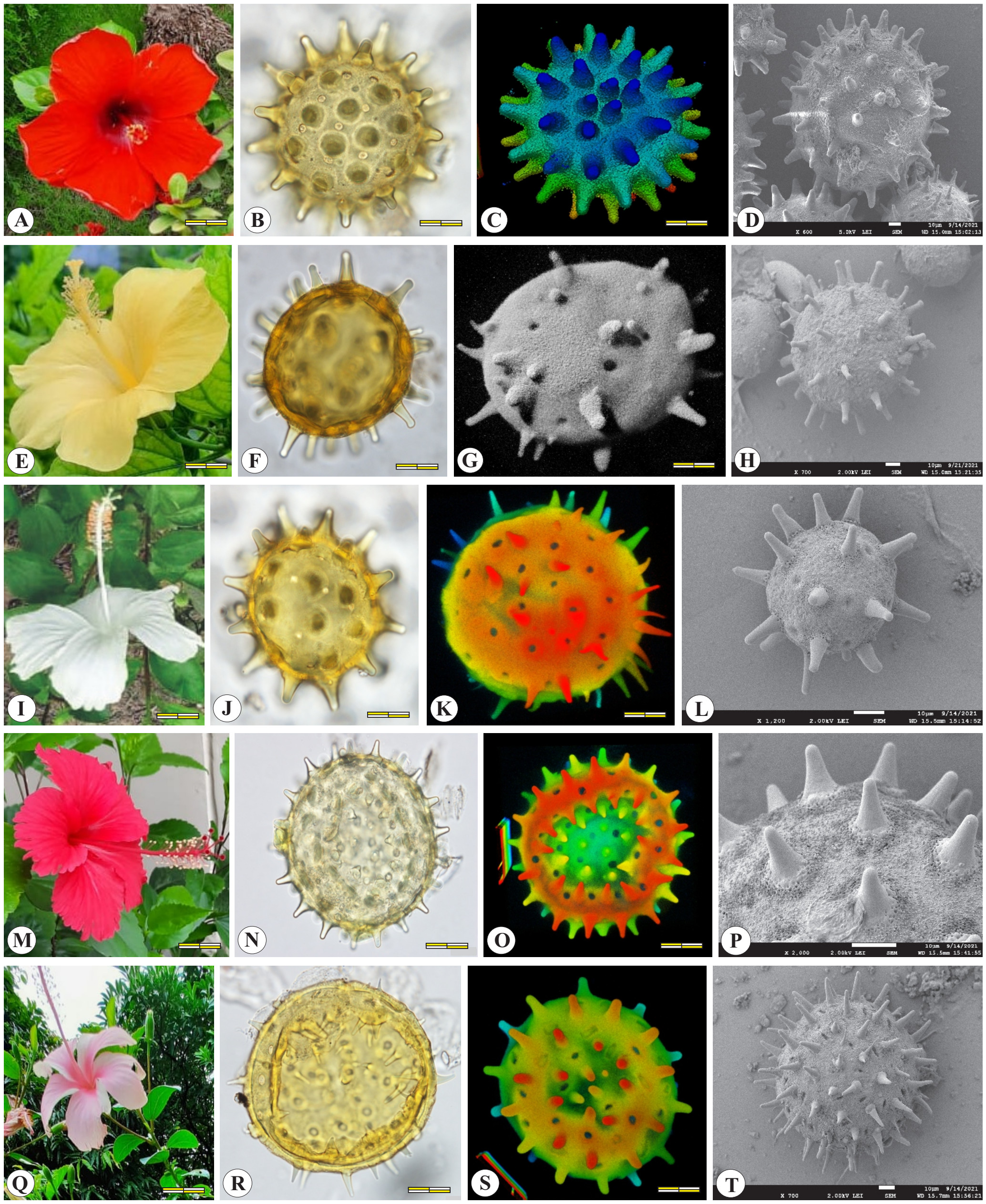

Fig. 2: LM, CLSM and FESEM images of the *H. rosa-sinensis* L. and hybrids (HRS H 1-HRS H 4).

L. H14, R. LM microphotograph; R. spines focussed showing distinct spines apices and bases, a few pores also visible, S. CLSM image: S. pores focussed along with exine and spines, surface features also seen, T. FESEM image; T. spines focussed in a complete grain, showing surface features with distinct perforations and minute granules.

Protocols for sample preparation and microscopy

The pollen grains were acetolysed and the slides were prepared in glycerine jelly for light microscopy (LM) following Erdtman (1943, 1954). The prepared reference pollen slides (Figs. 2-4) are deposited and stored in BSIP herbarium, Lucknow (Table 1). LM studies were carried out using an Olympus BX50 microscope with attached DP2 BSW Software for photography. For describing the *Hibiscus rosa-sinensis* L. hybrids, the shape, size/overall dimension, aperture (pores) number and size, as well as pollen wall architecture (exine thickness, pattern, etc.) were considered. Additionally, pollen symmetry, polarity, exine ornamentation, structure etc. were also statistically analyzed (Table 1; Figs. 2-4).

Confocal laser scanning microscopy *(CLSM)*

The pollen slides prepared for LM studies were used for capturing images of pollen in all 15 hybrids of *H. rosa-sinensis* through the CLSM. The study was carried out at the CLSM and Raman Spectroscopy Laboratory of the BSIP, Lucknow. The details of the protocol adopted for conducting the CLSM study and its importance have been provided in Quamar *et al.* (2017, 2021, 2022a, 2022b).

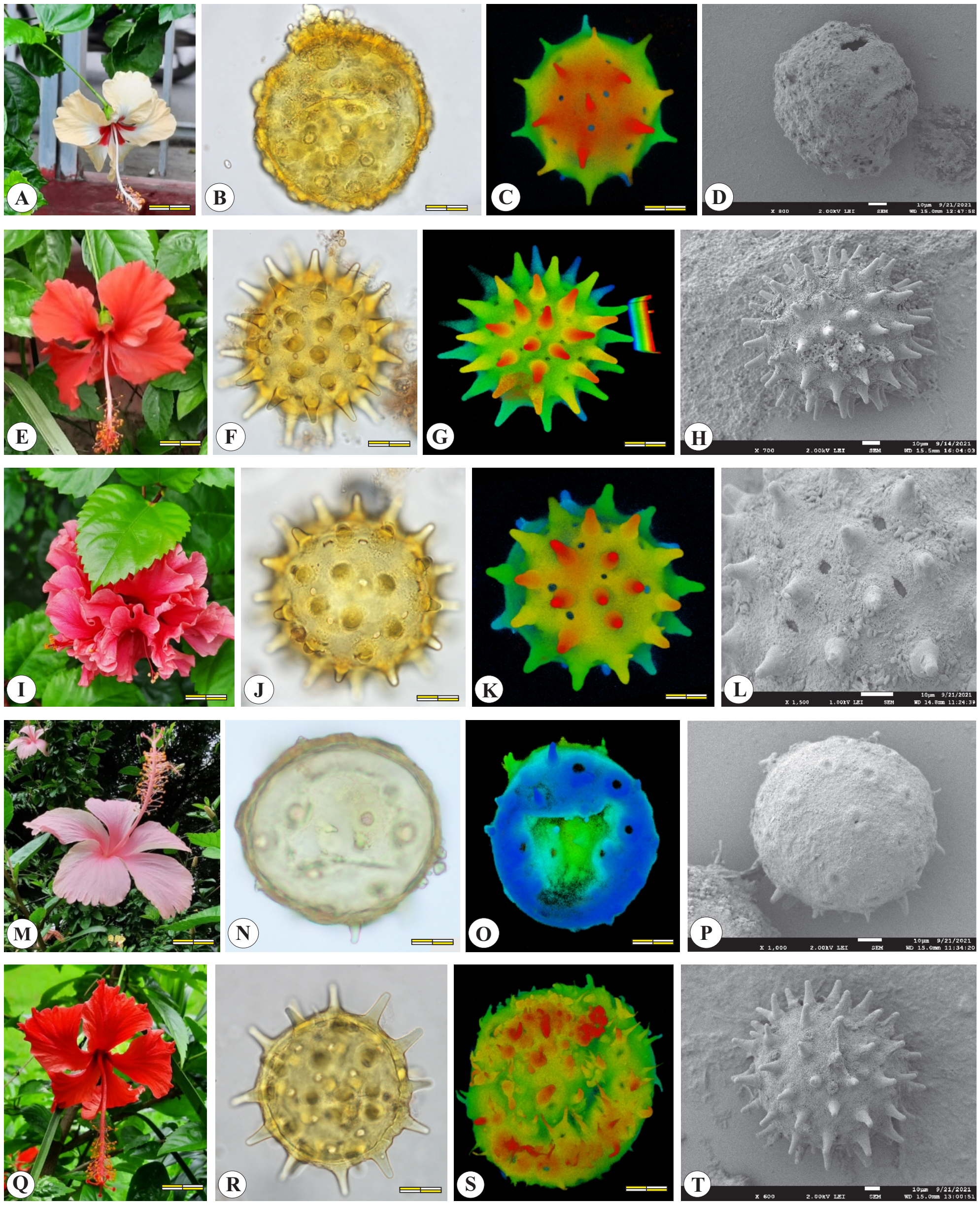

Fig. 3: LM, CLSM and FESEM images of the *H. rosa-sinensis* L. hybrids (HRS H5-HRS H9).

Field emission scanning electron microscopy *(FESEM)*

For the FESEM study, the anthers from the flowers were crushed in a beaker containing de-ionized water. Thereafter, the samples were fixed with 2.5% glutaraldehyde for 24 hours at room temperature. Washing of the samples with distilled water was done twice in order to remove the fixative. Subsequently, to dehydrate, the samples were given a series (10, 30, 50, 70, 90 and 100%) of ethanol treatment. The dried samples were then mounted on adhesive carbon tape placed on the copper stubs (Farooqui *et al.,* 2019). These prepared stubs were then coated by 70 s with palladium alloy (JEOL JEC 3000FC auto fine coater). Finally, the stubs were observed under high vacuum FESEM, JSM 7610f JEOL model and the images were taken at a desired magnification at 5 KV with LEI (lower electron imaging) and subsequently photographed.

Statistical analysis

The morphometry of pollen characters recorded under CLSM and FESEM were subjected to PCA to explore the numerical relationship among the 15 studied *Hibiscus rosa-sinensis* hybrids using PAST (version 3.24; Hammer *et al*., 2001). We used seven variables for the PCA, which include polar axis/polar length, equatorial diameter, pore length, pore width, spine length, spine width and spine base. The obtained results of PCA are shown in Tables 2 and 3 and also in Fig. 5.

Results

The pollen morphological characteristics and morphometry of 15 hybrids of *Hibiscus rosa-sinensis* L. (Malvaceae), including the species under consideration, using the LM, displayed the

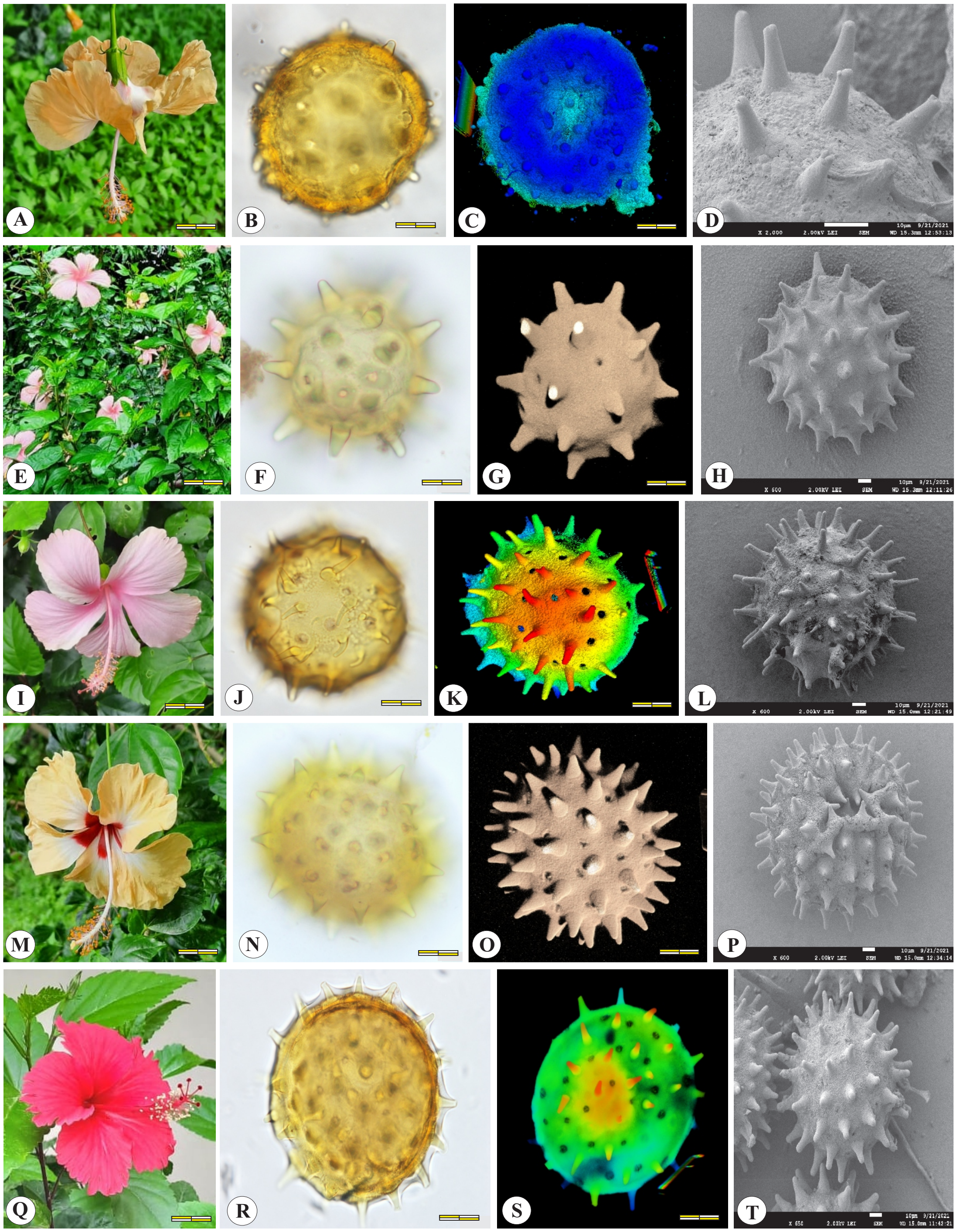

Fig. 4: LM, CLSM and FESEM images of the the *H. rosa-sinensis* L. hybrids (HRS H10-HRS H14).

variations in size, shape, aperture (pores) number, diameter and spine arrangement, exine thickness and diameter (pollen wall architecture), spinules, as well as spines length, their width, spine bases of spines, their nature (smooth and bifurcation), appearance (elongated and/or curved) and dispensation (Table 1). In addition, CLSM and FESEM images were also taken to look for clearer images and also the characters, which are not seen in the LM.

Statistical analysis

The PCA results, illustrated in Fig. 5, are described through Component 1 (PC1) and Component 2 (PC2). Moreover, the PCA results highlight the significance of similarities between various palyno-morphological traits along with PCA biplot with all the pollen traits in 15 hybrids of *Hibiscus rosa-sinensis* L. The extracted variance is revealed through a variance-covariance matrix and coordinates in a distance-based biplot. The obtained results of the PCA explained 98% of the variance with principal components 1 and 2, which significantly highlights the variability in morphometry of pollen grains observed in hybrids of *Hibiscus rosa-sinensis* L. hybrids (Tables 1-3; Fig.5) (Debut *et al*., 2013; Bahadur *et al*., 2022).

Discussion

Our results show that the morphometrical studies of pollen ultrastructure have a significant taxonomic potential to identify the *Hibiscus rosa-sinensis* L. hybrids. The shape of the pollen in all the hybrids of *Hibiscus* varied from prolate to spheroidal. Similar studies carried out in pollen with hybrids of *Gossypium* too show variability in spheroidal pollen shape (Cai *et al*., 2022). Pollen size too varied in *Gossypium*, which is very similar to our results with hybrids of *H. rosa-sinensis*.

The P/E ratio (ratio of the values of Polar diameter and Equatorial distance of pollen) largely varied from 35 to 70 µm within the hybrids. The smallest size was in H10 (35/33 µm) followed by H5, H10 and H11 (Table 1). The sizes of other pollen hybrids were larger, measuring 50-70 µm (H1- H5). Hence, it is evident that a large variation in size occurred in hybrids of *H.*

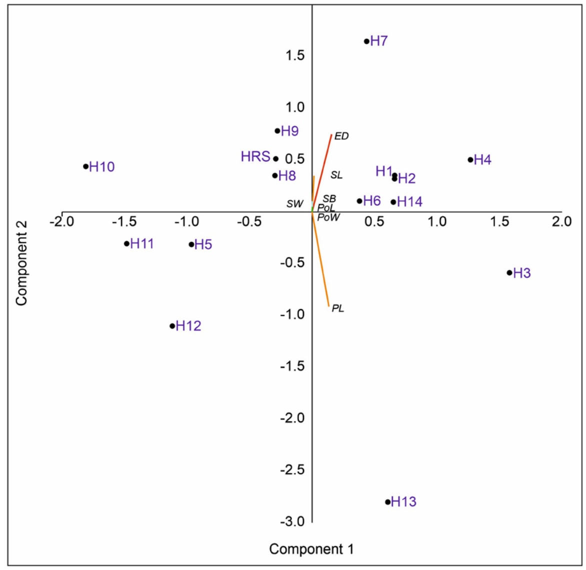

Fig. 5: Principal component analysis with seven metric variables of the studied hybrids of *Hibiscus rosa-sinensis* L. (Please see Table 1 for the abbreviations used in the PCA analysis).

Table 2: The eigenvectors and cumulative variance of PCA use the pollen quantitative traits of the *Hibiscus rosa-sinensis* L. hybrids.

S.No.	*Hibiscus rosa-sinensis L. hybrids*	*Axis 1*	*Axis 2*	*Axis 3*	*Axis 4*	*Axis 5*	*Axis 6*	*Axis 7*
1.	Hibiscus rosa- sinensis L. (HRS) Flower red color with yellow shade	-0.29	0.51	-0.28	0.45	0.32	1.02	1.06
2.	Hibiscus rosa- sinensis L. (HRS H1) Flower pure yellow and dark yellow color at the base	0.66	0.35	1.08	0.29	0.30	-1.95	2.62
3.	Hibiscus rosa- sinensis L. (HRS H2) Flower pure white	0.66	0.32	0.92	-0.50	0.05	0.18	0.22
4.	Hibiscus rosa- sinensis L. (HRS H3) Flower dark pink-shaded	1.58	-0.59	-1.39	-0.38	-0.66	0.71	-3.66
5.	Hibiscus rosa- sinensis L. (HRS H4) Pink flower with white shade	1.27	0.50	-1.14	-0.99	-0.63	-0.82	-4.30
6.	Hibiscus rosa- sinensis L. (HRS H5) Flower white creamy double and dark red color at the base	-0.96	-0.31	-0.13	0.19	1.46	-1.97	-0.29
7.	Hibiscus rosa- sinensis L. (HRS H6) Flower light peach in color	0.38	0.11	2.15	0.39	0.37	1.23	4.62
8.	Hibiscus rosa- sinensis L. (HRS H7) Flower scarlet-red double flowers with ruffled petals.	0.44	1.65	-0.26	2.29	-0.44	-0.25	7.07
9.	Hibiscus rosa- sinensis L. (HRS H8) Purple flower and light pink color at the base	-0.30	0.35	-1.12	-0.49	0.38	0.03	-3.09
10.	Hibiscus rosa- sinensis L. (HRS H9) Flower dark red in color	-0.28	0.78	0.98	-2.28	0.49	0.52	-4.36
11.	Hibiscus rosa-sinensis L. (HRS H10) Flower golden yellow and white color at the base	-1.81	0.44	-1.25	0.03	1.24	0.89	-2.00
12.	Hibiscus rosa- sinensis L. (HRS H11) Flower light pink in color and red color at the base	-1.48	-0.31	0.40	0.62	-2.02	0.46	3.04
13.	Hibiscus rosa- sinensis L. (HRS H12) Flower pink with dark pink shade at the base	-1.12	-1.10	0.19	-0.64	-1.84	-0.91	-1.12
14.	Hibiscus rosa-sinensis L (HRS H13) Flower dark yellow in color and white and dark red color at the base	0.61	-2.80	0.03	0.81	1.05	0.40	0.29
15.	Hibiscus rosa- sinensis L. (HRS H14) Flower deep pink in color and dark yellow color at the base	0.65	0.10	-0.17	0.20	-0.07	0.47	0.25

Table 3: The PCA analysis, based on quantitative traits of *Hibiscus rosa-sinensis* L. hybrids.

PC	*Eigenvalue*	*% variance*
1	225.97	95.68
2	6.30	2.67
3	2.66	1.13
4	1.01	0.43
5	0.16	0.07
6	0.06	0.03
7	0.00	0.00

rosa-sinensis plant. The pollen size varied in hybrids of *Gossypium* from ~60-103 µm. Moreover, a large variability in shape, number and length of exine echini shows variation within the *Gossypium* hybrids. The pollen size in *Sida* varies between 70 to 110 µm and the length of the shortest spine is 3 µm and the longest is 14 µm, which are diagnostic features within the genus (Agwu *et al*., 2015). Our results show that the pollen diameter of *H. rosa-sinensis* hybrids varied from 35- 70 µm and, therefore, can be distinguished at a generic level. Fukuda *et al*., were of the opinion that the increase in pollen size usually increases with the increase in chromosome number. The size of pollen grains in *Gossypium* is highly correlated to the length of exine spine, but it is less correlated to the number of spines (Cai *et al.,* 2022**)**. It has been observed that the longest spines are recorded in *Abelmoschus esculentus* (15 µm), and the shortest (3 µm) are in *Malva parviflora* (El Naggar and Sawady, 2008). In *H. rosa-sinensis,* the shortest spine length is 5 µm (rarely 3 µm) and longest is 10 µm, as documented among all the studied hybrids. Therefore, these morphometrical studies would help us to precisely identify pollen in sedimentary archives.

The rod-like echinae on exine can be differentiated into conical bulbous or non-bulbate bases in all 15 hybrids of *Hibiscus*. In one or two hybrids the echinae appear tuberculate. Similar echinae characters have been observed in *Gossypium* and are related to the E-genome (Cai *et al.,* 2022). The distance between the spines is also one of the distinguishing characters which is ~10-15 µm in *Hibiscus*, *Gossypium*, *Abelmoschus* and ~5 to 7 µm in pollen of *Malva* and *Alcea*. The pollen of *H. rosa sinensis* shows strong Fullerine-based symmetry with respect to the distribution of its spines over the spherical grains forming spherical hexagons and pentagons (Andrade *et al*., 2014). The distance between the spines in some hybrids (Fig. 4) does not show a perfect hexagon or pentagon, suggesting characteristic changes. Significant morphometrical changes in the size and sculpture of pollen have great taxonomic potential in the identification and delimitation of species (Ullah *et al.,* 2018). Our study reveals significant differences in pollen size and echini morphology even in LM and its ultrastructures, such as branching pattern and ornamentation of tecta was observed and measured in FESEM and CLSM, indicating that these characteristics can be a useful index for taxonomy at the species/subspecies level. Exine ornamentation also plays a significant role in taxonomy within the family, genus and at a specific level in the family Brassicaceae and Geranium family (Khalik *et al*., 2002; Erik, 2012; Baser *et al*., 2016). The length of spines in all the studied *Hibiscus rosa-sinensis* hybrids is 3 to 10 µm, whereas in *Abelmoschus esculentus* (15 µm) it is the longest. The shortest are in *Malva parviflora* (El Naggar and Sawady, 2008). These variations are of taxonomic value at different taxonomic levels, because they may occur not only between genera but also between species of the same genus.

The PCA analysis suggests that polar axis/polar length, equatorial diameter, pore length, pore width, spine length, spine width and spine base are the significant pollen morphological traits in hybrids. In the PCA biplot, the observed specimens of H1, H2, H6, H14, H4 and H7 resemble positive correlations with equatorial diameter (ED), spine length (SL), spine width (SW) and spine base (SB). Here, PoL and PoW is in positive correlation with H3 and H13 where the studied parameters and Euclidean space is larger, describing relatively higher diversity among these specimens. Morphological traits of HRS, H9 and H8 are in good correlation with respect to SW and SB. The observed H5, H10, H11 and H12 resemble negative correlation with respect to the studied morphological parameters. Similarly, the box and jitter plot (Fig. 6) showed that observed specimens with different colors represent diversity among the 15 studied *Hibiscus rosa-sinensis* L. hybrids. The x-axis represented the analyzed traits, i.e. polar axis/polar length, equatorial diameter, pore length, pore width, spine length, spine width and spine base, whereas the y-axis represented their measurement around mean values.

The genus *Hibiscus* includes many polyploid species, particularly *H. syriacus*, *H.aspera* and *H. rosa-sinensis* (Kim *et al*., 2017) and, hence, polyploidy influences on plant genome evolution is a very well-established phenomenon (Semon and Wolfe, 2007; Soltis *et al*., 2015). Although ploidy is expected to be an ‘evolutionary dead end’ to many species (Otto and Whitton, 2000), polyploidization of plants helped in the survival

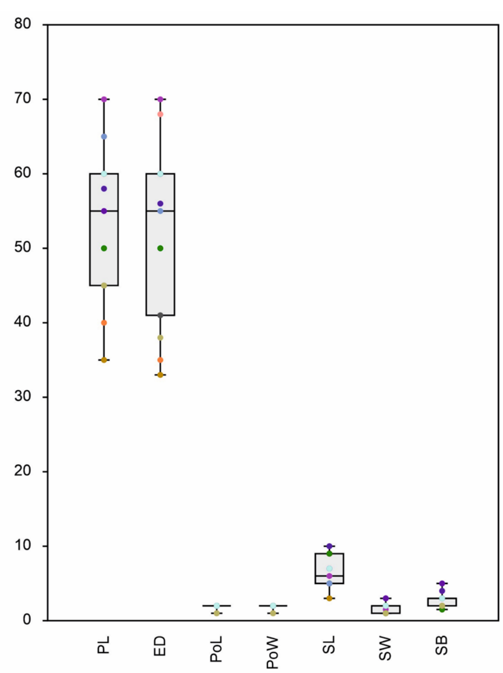

Fig. 6: Box plot and Jitter plot, showing the various hybrids of *Hibiscus rosa-sinensis* L. (the same color jitters showing the hybrids with same characteristics, whereas different colors represent the various hybrids of the taxa under study.

of plants, in general, during the Cretaceous-Tertiary extinction event by increasing their genetic diversity (Fawcett *et al*., 2009). Polyploidization results in interchromosomal rearrangements, neofunctionalization, and subfunctionalization (Semon and Wolfe, 2007; Soltis *et al*., 2015). In the family, Malvaceae, *Hibiscus* and *Gossypium* include polyploid species and have been successfully used for developing hybrids for ornamental and economic values. *Gossypium* has long been used for its fiber. Recently, *Hibiscus* (*H. cannabinus*) has been used for natural fiber production, putting it on the list of economic values of plants (Teixeira da Silva, 2021).

Cross-pollination accounts for maximum genetic variation and recombination producing new genotypes displaying variation in plant micro-morphology (Eckert *et al*., 2010). Our comprehensive results with pollen from 15 hybrids of *Hibiscus rosa-sinensis* is novel information to evaluate the pollen ultrastructure diversity within the same species and its taxonomic significance, which has altered due to cross-pollination, but the pollen morphometry correlates well with the color of the flower. Hence, pollen morphometry can allow the identification of the flower color of a hybrid plant. Significant variation in the size, shape, and length of the spine has been observed in *Hibiscus rosa-sinensis* hybrids, and, therefore, the LM and ultrastructure of pollen is important for identification in palynological studies and proper interpretations related to diversification/evolution in pollen either due to natural or anthropogenic reasons through a geological period.

Conclusion

The morphometry of pollen ultrastructure serves as a valuable taxonomic tool in plant systematics. The present comprehensive study of 15 hybrids of *Hibiscus rosa-sinensis* L. (Malvaceae) developed by cross-pollination and grown in Lucknow, India, in sub-tropical climate revealed large variation in micro-morphometrical characters of the pollen. The polar axis, equatorial diameter, aperture (pore) morphology and diameter, echinate ornamentations of exine, and its spine length, width and base have been measured using LM, CLSM and FESEM. The PCA analysis suggests that polar axis/polar length, equatorial diameter, pore length, pore width, spine length, and shape were significant pollen characters having potential in phylogenetic and taxonomic implications. We infer that pollen ultrastructure varied in all the hybrids with regard to pollen size, shape and echinate morphology. The pollen size is in direct correlation with the spine index. The smaller is the size of pollen the smaller is the spine index, which correlates well with other characters. Polyploidization in *Hibiscus* and *Gossypium* occurs in family Malvaceae and both show variations in the pollen morphology of hybrids. Our study, however, shows a larger variation in pollen morphometry of *Hibiscus* which can be identified among the hybrids and can relate quite well with the color of the flower developed through cross-pollination. The study facilitates the identification of pollen during palynological studies of past and present. This also provides a clue to index in systematics, evolution and plant taxonomy.

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Author contribution

Mohammad Firoze Quamar[1,2]* and Anjum Farooqui[1]* conceived the idea, collected the samples and involved in original draft preparation. Nagendra Prasad[1,2], Salman Khan[1], Mohammad Javed[1] and Maneesha M ET[1,2] processed the samples for LM, CLSM and FESEM studies, prepared the plates, and done statistical analysis. Mohammad Firoze Quamar[1,2]*, Salman Khan[1] and Anjum Farooqui[1]* done the LM studies, generated the data and taken the LM microphotographs. Nagendra Prasad[1,2], Mohammad Javed[1] and Maneesha M ET[1,2] taken the CLSM and FESEM microphotographs. The final editing was done by Mohammad Firoze Quamar[1,2]* and Anjum Farooqui[1]*, and also supervised the work. All authors read and approved the final version of the manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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