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Pollen morphological study in subfamily Papilionoideae using Confocal Laser Scanning Microscopy

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ABSTRACT

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The pollen morphological study was carried out in the subfamily Papilionoideae using Confocal Laser Scanning Microscopy (CLSM) to facilitate the identification of pollen in sedimentary archives. Pollen has long been used as an excellent proxy for understanding past vegetation, ecology, climate and agricultural strategies of ancient settlements and therefore, its identification at a specific level is of utmost importance. The modern pollen samples were retrieved from plants growing in urban and rural areas of Kanpur city, Uttar Pradesh, India. The cluster analysis and PCA of pollen morphological characters in the subfamily determine the generic and species relationships outlining the affinity of taxa in subfamily Papilionoideae.

Key–words—CLSM, Pollen morphology, Papilionoideae, Kanpur, India, Cluster Analysis.

INTRODUCTION

FABACEAE (Leguminosae) is the third largest family of angiospermous plants comprising about 11% of the known legume flora with 657 genera and 16400 species reported by Rendle (1952), Mabberley (1987) and Ferguson and Skvarla (1983). The subfamily Papilionoideae is eurypalynous and economically important as a source of nutritious food, fibre, shelter, valuable medicines and also virulent poisons (Datta & Mukherji, 1952). It is common in the tropics and comprises about 50% of the plants, including herbs, shrubs and trees. Morphological characters of pollen have been used for a long to resolve the taxonomic issues within genera and species in subfamilies. Pollen grains are different in size, shape, symmetry, colpi, aperture, and exine ornamentation. These characters or pollen traits provide a better understanding of their taxonomic relationships (Bhattacharya *et al.*, 2009). The subfamily Papilionoideae is characterized by its distinct Papilionaceous flower with tricolporate pollen as described by Fritzsche, 1832 and Fischer, 1890. Subsequently, several workers, viz. Von Mueller (1876), Ikuse (1956), Faegri and Iversen (1975), Perveen and Qaiser (1998), Masood *et al*., (2015), and Bano *et al*. (2018) described them from different parts of the globe.

Owing to the small size of the pollen grain, the acquisition of pollen morphological information under Light Microscopy is difficult. Hence, the pollen morphological studies are scant with images of a Light microscope (Vishnu–Mittre & Sharma, 1962; Nair, 1970, 1990). Therefore, Confocal Laser Scanning Microscope (CLSM) provides new insights into the precise identification of pollen at specific level in palynological slides. The utility of pollen identification of various plants could be of great help in plant taxonomy and systematics in order to understand the affinities of modern plant groups/families, and in ascertaining the definitive status of plants in various taxonomic groups (Mao *et al*., 2012; Gosling *et al*., 2013; Yang *et al*., 1998, 2015).

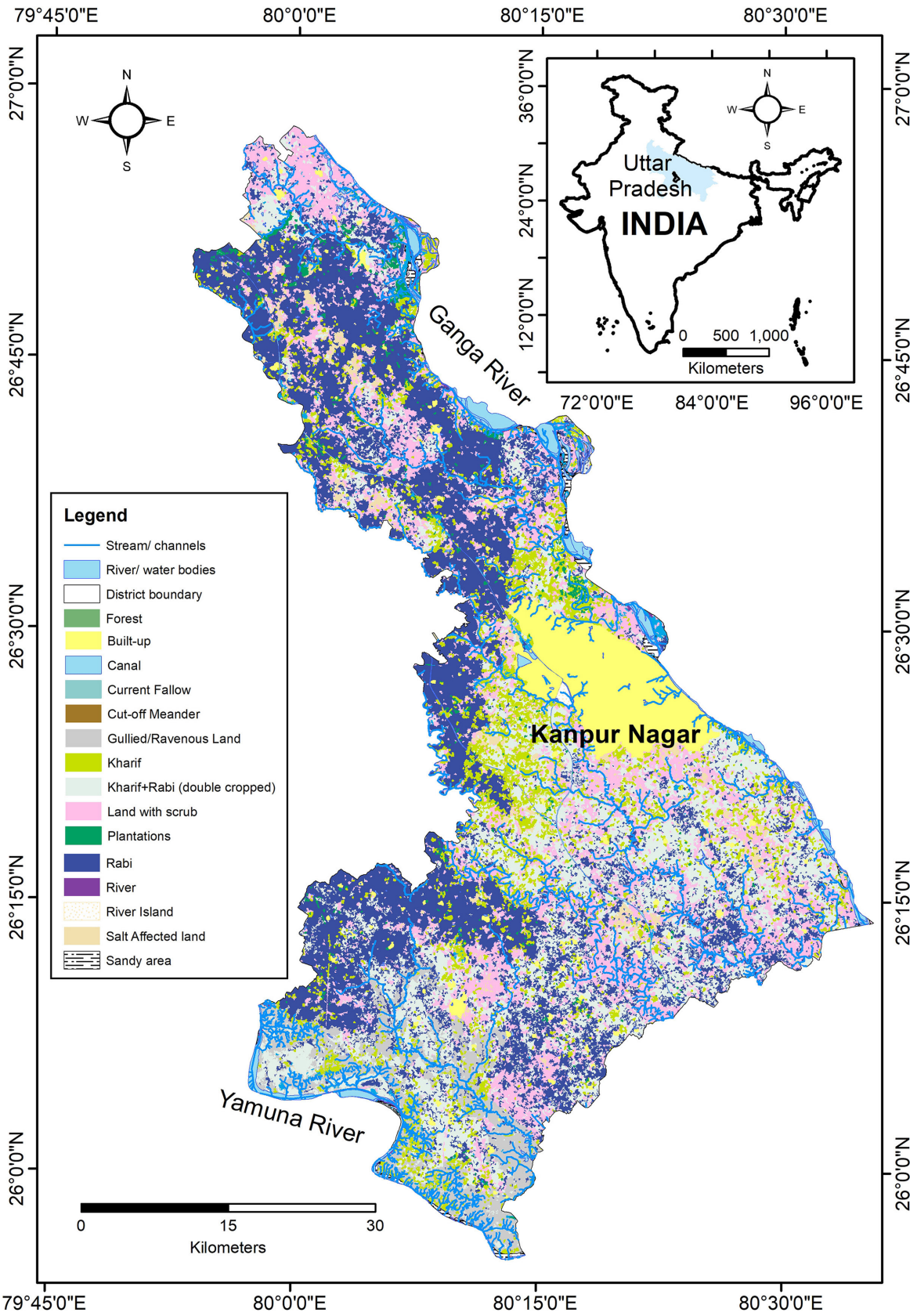

Fig. 1—Digital elevation model (DEM) with present study area (Kanpur), Uttar Pradesh, India.

The palynological study involves the identification of pollen using a traditional transmitted light microscope (LM) to understand palaeovegetation, palaeoecology and palaeodiets of humans and domestic animals (Yao *et al*., 2012; Tang *et al*., 2014; Basumatary *et al*., 2020). Several members of the subfamily Papilionoideae are a good source of food grain since ancient times (Sharma *et al*., 2021). The pollen morphological characters of these are not very distinguishable under Light Microscope and are in general grouped either under subfamily or generic level which fails to provide any clue related to the climate–culture relationship. The CLSM study in permanent palynological slides, on the other hand, makes it easier to see high–resolution images of species that are different within the same genus (Gavrilova *et al.,* 2018). We, therefore, attempted to describe the pollen morphology and morphometry of the 15 species under 14 genera (Table 1) of subfamily Papilionoideae using LM and CLSM contributing significant pollen characters of taxonomic importance. The statistical analysis of pollen characters also serves as a modern analogue for identifying fossil pollen preserved in sedimentary archives to infer the climate–vegetation relationship.

STUDY AREA

The flowers were collected from Kanpur District (Fig. 1) which lies in the sub–tropical climate in the Indian sub–continent. The study area is the largest fertile alluvial plain of the Ganga River with agricultural activities (Trivedi *et al*., 2013, 2019; Saxena *et al*., 2015). It covers an area of about 1,640 sq. km. The study area is located at 26º28'N latitude and 80º24'E longitude at an altitude of 126 meters above mean sea–level. Most of the area in Kanpur and its adjoining districts is under intensive cultivation of conventional crops along with arboriculture practices. The climate, in general, is humid sub–tropical and receives most of the rainfall through the southwest monsoon. Monsoon season begins in mid–June and continues till mid–September with an average rainfall of 1020–1140 mm. About 75% rainfall occurs during the monsoon season. The winter season starts from November to February and is characterized by average minimum and maximum temperatures of 7.6ºC and 21ºC, respectively (Trivedi *et al*., 2019). The average minimum and maximum summer temperatures are 27ºC and 32.5ºC respectively.

MATERIAL AND METHODS

Pollen analysis

For the present investigation, the polliniferous material was collected in separate well–labelled plastic vials from mature flower buds of well–identified plants growing in the Kanpur area (U.P.). The flower buds were soaked and gently crushed in distilled water and sieved with 150 µm mesh. The filtrate was acetolysed following Erdtman (1969). The acetolysed samples (Pls 1, 2) were mounted in glycerine jelly for preparing permanent palynological slides for examination under LM and CLSM. The LM observations of pollen morphological characters were made with an Olympus BX 50 linked to DP2–BSW software available at BSIP, Lucknow. The pollen of Papilionoideae was examined using conventional techniques (Faegri & Iversen, 1975) at the BSIP, Lucknow, for morphological characteristics at the submicron level. On a Leica TCS SP8 Confocal Laser Scanning microscope, three–dimensional confocal fluorescence (Gavrilova, 2012) imaging was obtained using two Melles Griot lasers, one at 488 nm (20mW output) and the other at 633 nm (10mW output) (Melles Griot, Carbsbad CA). An oil–immersion objective with a field of view of 100 mm was used to capture the photographs (f/1.4). The specimen's kerogen–derived fluorescence was reduced by filters and excitation at 488 nm. To compare the recorded autofluorescence, same conditions were employed for each acquisition. LAS–X imaging software was used to analyze and examine the collected images.

Pollen surface ornamentation and other morphological features were analysed using a confocal zoom magnification setting of 2 (94 nm/pixel) to capture an image of entire pollen grain surfaces. By averaging a few different frames, we were able to lessen the amount of image noise. When the best pollen grain resulted in the best channel selection, the signal to noise was improved by averaging over eight repetitions for each optical segment. The specimen's size and the requisite level of precision (0.2–0.5 mm) were taken into account while determining the distances between sections. Each type of pollen was assigned a pseudocolor to distinguish species and variations.

The morphological description of the pollen grains was done following Erdtman (1969). The shape classes, the ratio of polar axis and equatorial diameter, size classes, polar and equatorial outlines, number and position of apertures, measurements of aperture, mesocolpia, apocolpia, exine thickness, exine stratification, exine sculpturing, etc. were recorded (Tables 1–2). Each measurement is based on 12–15 pollen grains of each species.

Statistical analysis

The mean is given in parenthesis between the lowest and highest values. The numerical analysis, such as principal component analysis (PCA) and cluster analysis was carried out on 15 species using quantitative and qualitative pollen characters (Tables 1–2). The standard variables were employed for multivariate statistical analysis. Cluster analysis (CA) was done with the average taxonomic distance (i.e. Euclidean distance matrix) and unweighted pair–group method with arithmetic mean (UPGMA) clustering procedures using PAST3 (Hammer *et al.*, 2001). The cophenetic correlation coefficient was determined to discover the extent to which the clusters fit the distance matrix. Further PCA was conducted

Table 1—Quantitative pollen morphological data of the observed species of Papilionoideae subfamily; numbers (where required) refer to the minimum (mean) maximum.

Species	P (Polar Axis)	E = D (Equatorial Axis)	Cl L (Colpi Length) / Pore Length*	Cl W (Colpi Width) / Pore Width*	Msp (Mesocolpia) / Mesoporiam^	Apc = d (Apocolpia) / Apoporium^
1. *Butea monosperma*	32 (32.5) 33	34 (34.5) 35	28 (28.50) 29	4 (4.5) 5	20 (21) 22	1 (1.75) 2
2. *Cajanus cajan*	30 (30.7) 32	32 (33.33) 36	18 (18.67) 20	2 (3) 3.5	12 (14) 16	6 (6.33) 7
3. *Cicer arientum*	24 (25.3) 26	21 (23) 24	18 (19.33) 20	2(2.17) 2.5	6 (7) 8	3 (3.17) 3.5
4. *Clitoria ternatea*	26 (31) 36	56 (61.33) 70	18 (21) 24*	14 (14) 16*	20 (22.6) 26^	4 (5) 6^
5. *Crotalaria juncea*	21 (22.3) 24	12 (13.33) 14	18 (18.67) 20	2 (2.17) 2.5	3 (4.33) 5	1.5 (1.83) 2
6. *Crotalaria retusa*	22 (22.7) 24	16 (18) 20	16 (17.33) 18	2 (2.33) 2.5	5 (6) 7	2 (2.67) 3
7. *Dalbergia sissoo*	20 (21.3) 22	19 (20) 21	10 (12) 14	2(2.5) 3	8 (9.33) 12	3 (4.67) 6
8. *Erythrina stricta*	16 (17) 18	24 (25) 26	4 (5) 6*	4 (5) 6*	7 (7.5) 8^	5 (5.5) 6^
9. *Lablab purpureus*	34 (36) 38	32 (32.67) 34	24 (25.33) 28	1.5 (1.67) 2	14 (15) 16	5 (5.33) 6
10. *Millettia ovalifolia*	16 (18) 20	17 (18.5) 20	12 (14) 16	1.5 (1.83) 2	8 (9) 10	1.5 (1.75) 2.5
11. *Lathyrus oleraceus*	42 (43) 44	20 (21.33) 22	30 (32) 34	1.5 (2) 2.5	8 (10) 12	4 (5.33) 6
12. *Pongamia pinnata*	22 (23.5) 24	22 (26) 31	18 (19.00) 20	1.5 (1.8) 2	16 (17) 18	1 (2) 3
13. *Trifolium alexandrinum*	30 (31.3) 34	22 (23.33) 26	24 (25.33) 28	2 (2.33) 2.5	12 (14) 16	3 (3.33) 3.5
14. *Trigonella foenum–graecum*	22 (25) 28	16 (17.5) 20	18 (18.50) 20	1 (1.25) 1.5	8 (10) 12	2 (3) 4
15. *Vicia sativa*	34 (36) 38	24 (24.67) 26	22 (23.33) 24	1.5 (1.83) 2	16 (17) 18	5 (6) 7

on a total of 15 pollen characters (Table 3) with commercially available Minitab statistical software (Ryan & Joiner, 2001) and Origin Pro.

Based on different pollen characteristics, the total PCA Score is 73%, whereas PC1 and PC2 variation is 41.9 and 21.6% respectively (Table 4).

RESULTS

Descriptive morphology of Pollen grains

The CLSM investigation of pollen has provided a significant high image resolution for pollen identification. Pollen grains of the Papilionoideae showed variation in their morphological characters. The detailed collective results obtained from LM and CLSM for Papilionoideae pollen are given in Table 1 to 4 and Pls 1–7. Pollen characteristics for studied taxa are described below.

Butea monosperma Lam. Kuntze, Common name: Flame–of–the–forest

(Pl. 1.1–8)

Pollen grains oblate spheroidal, P/E 0.93 (0.94) 0.95, ME, 32.0 (32.5) 33.0 µm x 34.0 (34.5) 35.0 µm, polar outline subtriangular, equatorial outline elliptical; trizonocolporate. Colpi broad, linear (28.0 (28.5) 29.0 µm long) and wide (4.0 (4.5) 5.0 µm), protruding at the equator, tips blunt, margins smooth. Ora lalongate, length (6.0 (6.5) 7.0 µm long) and width (8.0 (9.0) 10.0 µm) at equator elliptical, annulate. Mesocolpia 20.0 (21.0) 22.0 µm, apocolpia 1.5 (1.75) 2.0 µm across. Exine 2.0 µm thick, sexine (1.0 µm) as thick as nexine (1.0 µm) or slightly thicker, sculpturing reticulate, homobrochate, brochi medium; tegillate.

Cajanus cajan L. Millsp., Common name: Pigeon pea

(Pl. 1.9–15)

Pollen grains oblate spheroidal, P/E 0.89 (0.92) 0.94, ME, 30.0 (30.67) 32.0 µm x 32.0 (33.33) 36.0 µm, polar outline triangular, equatorial outline elliptical; trizonocolporate. Colpi short, narrow, linear, 18.0 (18.67) 20.0 µm long, 2.0 (3.0)

Table 1—(continued)

Species	P/E	Ex (Exine)	Sx) (Sexine)	Nx (Nexine)	Ratio d/D	Ratio Cl L/P	O Lo (Ora Length) / Pore length~	O Wi (Ora Width) / Pore Width~
1. *Butea monosperma*	0.93 (0.94) 0.95	2	1	1	0.05	0.88	6 (6.05) 7	8 (9) 10
2. *Cajanus cajan*	0.89 (0.92) 0.94	3	1.5	1.5	0.19	0.61	5 (5.67) 6	6 (6.33) 7
3. *Cicer arientum*	1.08 (1.10) 1.14	2	1	1	0.14	0.76	10 (10.17) 10.5	8 (8.67 10
4. *Clitoria ternatea*	0.46 (0.48 0.51	3.5	2	1.5	0.00	0.00	18 (20) 24~	14 (14) 16~
5. *Crotalaria juncea*	1.50 (1.68) 1.83	1.75	1	0.75	0.14	0.84	3.5 (4) 4.5	6 (7) 8
6. *Crotalaria retusa*	1.20 (1.27) 1.38	2	1	1	0.15	0.76	3.5 (3.75) 4	7.5 (8) 8.5
7. *Dalbergia sissoo*	1.00 (1.07) 1.10	2	1	1	0.23	0.56	4 (5) 6	8 (9.33) 10
8. *Erythrina stricta*	0.62 (0.68) 0.72	2	1	1	0.00	0.00		
9. *Lablab purpureus*	1.06 (1.10) 1.13	3	2	1	0.16	0.70	5 (5.33) 6	5 (5.33) 6
10. *Millettia ovalifolia*	0.90 (0.95) 1.00	2	1	1	0.09	0.78	4 (4.67) 6	8 (9.33) 10
11. *Lathyrus oleraceus*	1.91 (2.00) 2.10	2	1	1	0.25	0.75	6 (6.33) 7	7 (7.33) 8
12. *Pongamia pinnata*	0.77 (0.92) 1.09	1.85	0.62	1.25	0.08	0.81	4 (4.25) 4.5	5 (5.5) 6
13. *Trifolium alexandrinum*	1.31 (1.34) 1.36	2	1	1	0.14	0.81	4 (4.67) 6	6 (7) 9
14. *Trigonella foenum–graecum*	1.30 (1.41) 1.56	2	0.5	1.5	0.17	0.76	4 (5) 6	6 (6.25) 6.5
15. *Vicia sativa*	1.42 (1.43) 1.46	2	1	1	0.24	0.66	4 (5) 6	4 (5) 6

3.5 µm wide at equator, tips acute, and margins smooth. Ora circular to lalongate, 5.0 (5.67) 6.0 µm long, 6.0 (8.33) 10.0 µm wide at equator, annulate. Mesocolpia 12.0 (14.0) 16.0 µm, apocolpia 6.0 (6.33) 7.0 µm across. Exine 3.0 µm thick, sexine (1.5 µm) as thick as nexine (1.5 µm) or slightly thicker, sculpturing distinctly reticulate, heterobrochate and tegillate.

Cicer arietinum L., Common name: Chick–pea

(Pl. 2.1–7**)**

Pollen grains prolate spheroidal, P/E 1.08 (1.10) 1.14, ME, 24.0 (25.33) 26.0 µm x 21.0 (23.0) 24.0 µm, polar outline subtriangular, equatorial outline slightly elliptical; trizonocolporate. Colpi narrow, linear, 18.0 (19.33) 20.0 µm long, 2.0 (2.17) 2.5 µm wide and protruding at equator, tipsblunt with smooth margins. Ora circular to lolongate, 10.0 (10.17) 10.5 µm long, 8.0 (8.67) 10.0 µm wide at equator. Mesocolpia 6.0 (7.0) 8.0 µm, apocolpia 3.0 (3.17) 3.5 µm across. Exine 2.0 µm thick, sexine (1.0 µm) as thick as nexine (1.0 µm) or nexine slightly thicker, sculpturing faintly reticulate and tegillate.

Clitoria ternatea L., Common name: Conch flower

(Pl. 2.8–11)

Pollen grains peroblate to euoblate, [P/E 0.46 (0.48) 0.51, MA, 26.0 (30.0) 36.0 µm x 56.0 (61.33) 70.0 µm], polar outline subtriangular, equatorial outline elliptical; trizonoporate. Apertures large, elliptical and meridionally elongated, 18.0 (20.0) 24.0 µm long, 12.0 (14.0) 16.0 µm wide at equator. Mesoporia 20.0 (22.67) 26.0 µm, apoporia 4.0 (5.0) 6.0 µm across. Exine 3.5 µm thick, sexine (2.0 µm) slightly thicker than nexine (1.5 µm) and sculpturing psilate.

Crotalaria juncea L., Common name: Sun–hemp

(Pl. 2.12–14; Pl. 3.1–3)

Table 2—Species grouped according to the ornamentation; a rank is also assigned to each of the species.

Species	Sculpturing Type		
Type i			
Lablab purpureus	Crassisexinous and Faintly Reticulate	Tegillate	**0**
Type ii			
Cicer Arientum	Faintly Reticulate	Tegillate	**1**
Crotalaria juncea L.			
Crotalaria retusa L.			
Dalbergia sissoo Roxb.			
Millettia ovalifolia			
Pongamia pinnata			
Type iii			
Cajanus cajan	Reticulate, Heterobrochate, Brochi of	Tegillate	**2**
Erythrina stricta Roxb.	Variable Size and Shape		
Lathyrus oleraceus L.			
Trifolium alexandrinum L.			
Type iv			
Butea monosperma	Reticulate and Homobrochate	Medium Brochi & Tegillate	**3**
Trigonella foenum–graecum L.		Small Brochi & Tegillate	**4**
Type v			
Clitoria ternatea L.	Psilate		**5**
Vicia sativa L.			

Pollen grains euprolate, P/E 1.5 (1.68) 1.83, MI, 21.0 (22.33) 24.0 µm x 12.0 (13.33) 14.0 µm, polar outline subtriangular, equatorial outline sub rectangular; trizonocolporate. Colpi very long, narrow, linear, [18.0 (18.67) 20.0 µm long, 2.0 (2.17) 2.5 µm] wide at equator with acute tips and smooth margins. Ora lalongate, 3.5 (4.0) 4.5 µm long, 6.0 (7.0) 8.0 µm wide at equator, extended with indistinct margin. Mesocolpia 3.0 (4.33) 5.0 µm, apocolpia 1.5 (1.83) 2.0 µm across. Exine 1.75 µm thick, sexine (1.0 µm) slightly thicker than nexine (0.75 µm), sculpturing faintly reticulateand tegillate.

Crotalaria retusa L., Common name: Rattle weed

(Pl. 3.4–10)

PLATE 1

(Scale Bar: LM=20 µm, CLSM=100 µm)

1–8. *Butea monosperma* (1). Flower, (2–4). LM microphotographs, (2–3). Polar view, (4). Equatorial view showing trizonocolpate condition, colpi and reticulate ornamentation, (5–8). CLSM microphotographs, (5). Broad facet general view with colpus depth, (6). Overview with aperture, (7). Polar view with ornamentation and colpi with endoapertures, (8). Equatorial view of colpi with endoapertures and ornamentation.

9–15. *Cajanus cajan* (9). Flower, (10–12). LM microphotographs (objective 60 x), (10). Polar view, (11–12). Equatorial view with colpi, with endoapertures and ornamentation focused. (13–15). CLSM microphotographs, (13). 3D image of reticulate ornamentation along with colpus ridge, (14). Equatorial view showing trizonocolpate condition with prominant reticulate ornamentation, (15). Polar view showing position of apertures and ornamentation.

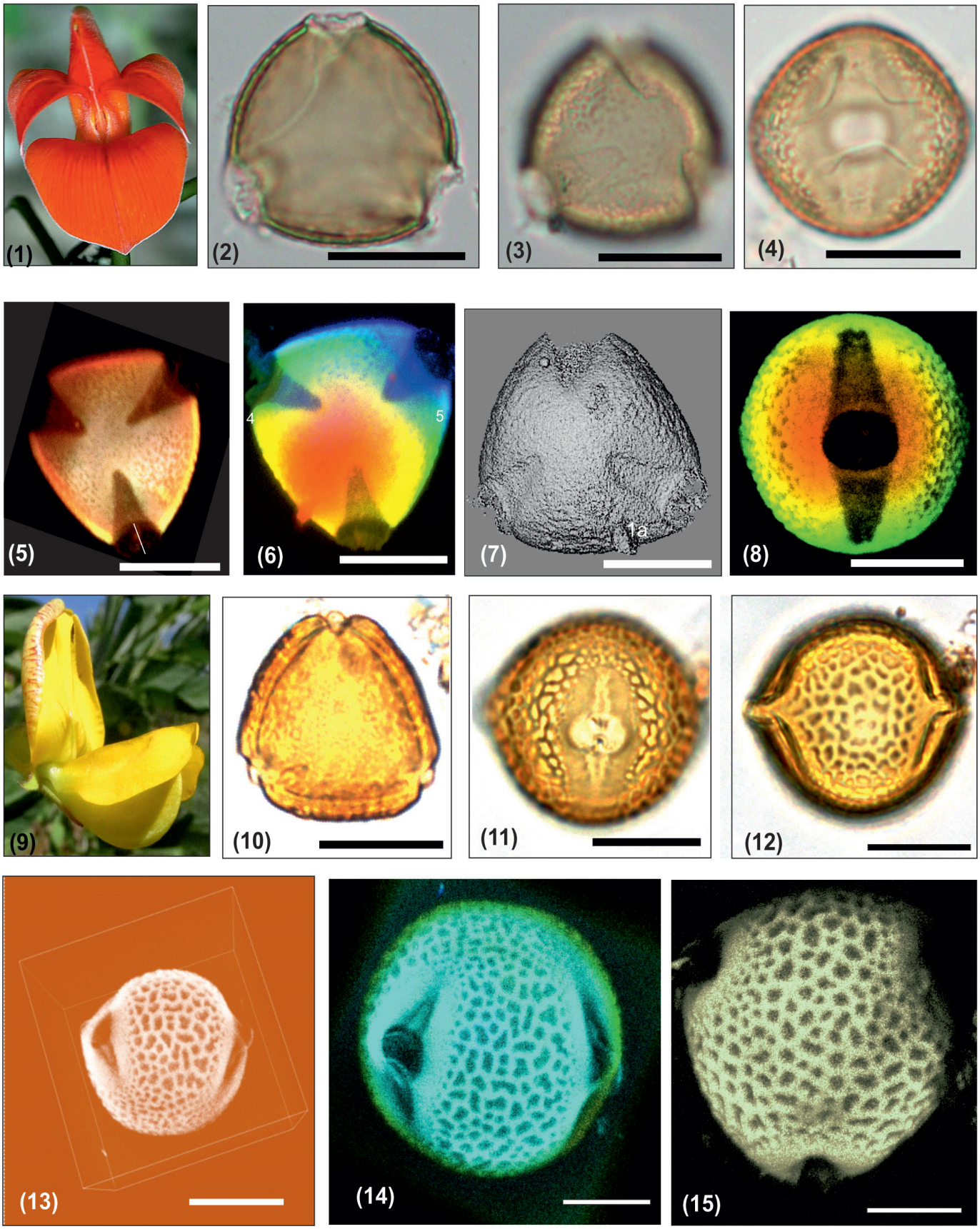

PLATE 1

Table 3—Variables of the loading scores against the first three principal components of 15 species of the Papilionoideae in the Kanpur region. Varying characters observed like P (Polar Axis), E = D (Equatorial Axis), Cl L (Colpi Length), Cl W (Colpi Width), Msp (Mesocolpia), Apc = d (Apocolpia), Ex (Exine), Sx (Sexine), Nx (Nexine), O Lo (Ora Length), O Wi (Ora Width) were studied and the dominant coefficients for PC1, PC2, and PC3 score are highlighted.

Variable	PC1	PC2	PC3
P	0.067	0.483	0.138
E (=D)	-0.326	0.28	-0.027
P/E	0.247	-0.076	0.537
Cl L	0.316	0.281	-0.006
Cl W	0.254	0.18	-0.157
Msp	0.235	0.303	-0.121
Apc (d)	0.219	0.363	-0.004
OLo	-0.334	0.154	0.185

Pollen grains subprolate to euprolate, [P/E 1.20 (1.27) 1.38, MI, 22.0 (22.67) 24.0 µm x 16.0 (18.0) 20.0 µm], polar outline subtriangular, equatorial outline elliptical; trizonocolporate. Colpi narrow, linear, 16.0 (17.33) 18.0 µm long, 2.0 (2.33) 2.5 µm wide at equator, tips blunt, margins smooth. Ora dumb–bell shaped, lalongate, 3.5 (3.75) 4.0 µm long, 7.5 (8.0) 8.5 µm wide at equator. Mesocolpia 5.0 (6.0) 7.0 µm, apocolpia 2.0 (2.67) 3.0 µm across. Exine 2.0 µm thick, sexine (1.0 µm) as thick as nexine (1.0 µm) or slightly thicker, sculpturing faintly reticulate; tegillate.

Dalbergia sissoo Roxb. ex DC., Common name: Sissoo

(Pl. 3.11–15)

Pollen grains spheroidal to prolate spheroidal, P/E 1.0 (1.07) 1.10, MI, 20.0 (21.33) 22.0 µm x 19.0 (20.0) 21.0 µm, polar outline subtriangular, equatorial outline circular to slightly elliptical; trizonocolporate. Colpi short, narrow and linear, 10.0 (12.0) 14.0 µm long, 2.0 (2.5) 3.0 µm wide at equator with acute tips and, smooth margins. Ora lalongate, large, 4.0 (5.0) 6.0 µm long, 8.0 (9.33) 10.0 µm wide at equator, sometimes slightly constricted in the middle. Mesocolpia 8.0 (9.33) 12.0 µm, apocolpia 3.0 (4.67) 6.0 µm across. Exine 2.0 µm thick, sexine (1.0 µm) as thick as nexine (1.0 µm), sculpturing faintly reticulate; tegillate.

Table 4—Eigenvalues of the Correlation Matrix showing affinity of different PCA against the studied qualitative and quantitative morphological characters.

	PC1	PC2	PC3
Eigenvalue	6.2803	3.2458	1.5511
Proportion	0.419	0.216	0.103
Cumulative	0.419	0.635	0.738

Erythrina stricta Roxb., Common name: Indian coral tree

(Pl. 4.1–6)

Pollen grains euoblate, [P/E 0.62 (0.68) 0.72, ME, 16.0 (17.0) 18.0 µm x 24.0 (25.0) 26.0 µm], polar outline triangular, equatorial outline elliptical and trizonoporate. Apertures are circular, 4.0 (5.0) 6.0 µm in diameter, annulate. Mesoporia 7.0 (7.5) 8.0 µm, apoporia 5.0 (5.5) 6.0 µm across. Exine 2.0 µm thick, sexine (1.0 µm) as thick as nexine (1.0 µm) or slightly thicker, sculpturing reticulate, heterobrochate and tegillate.

Lablab purpureus L., Common name: Hyacinth bean

(Pl. 4.7–13)

Pollen grains prolate spheroidal, P/E 1.06 (1.10) 1.13, ME, 34.0 (36.0) 38.0 µm x 32.0 (32.67) 34.0 µm, polar outline subtriangular, equatorial outline slightly elliptical; trizonocolporate. Colpi narrow, linear, 24.0 (25.33) 28.0 µm

PLATE 2

(Scale Bar: LM=20 µm, CLSM=100 µm)

1–7. *Cicer arietinum* (1). Flower, (2–4). LM microphotographs, (2). General view, (3). Equatorial view with colpi, aperture position and ornamentation, (4). Equatorial view showing ornamented colpi, (5–7). CLSM microphotographs, (5). Surface view showing ornamentation, (6). Polar view focussed on apertures, (7). Optical section and aperture focussed in different colours.

8–11. *Clitoria ternatea* 8. Flower, (9). LM microphotograph, general polar view, (10–11). CLSM microphotographs, (10). Surface view and aperture focussed, (11). Surface view pattern focussed with different colour shades.

12–14. *Crotalaria juncea* (12). Flower, (13–14). LM microphotograph, Overview showing colpi and ornamentation.

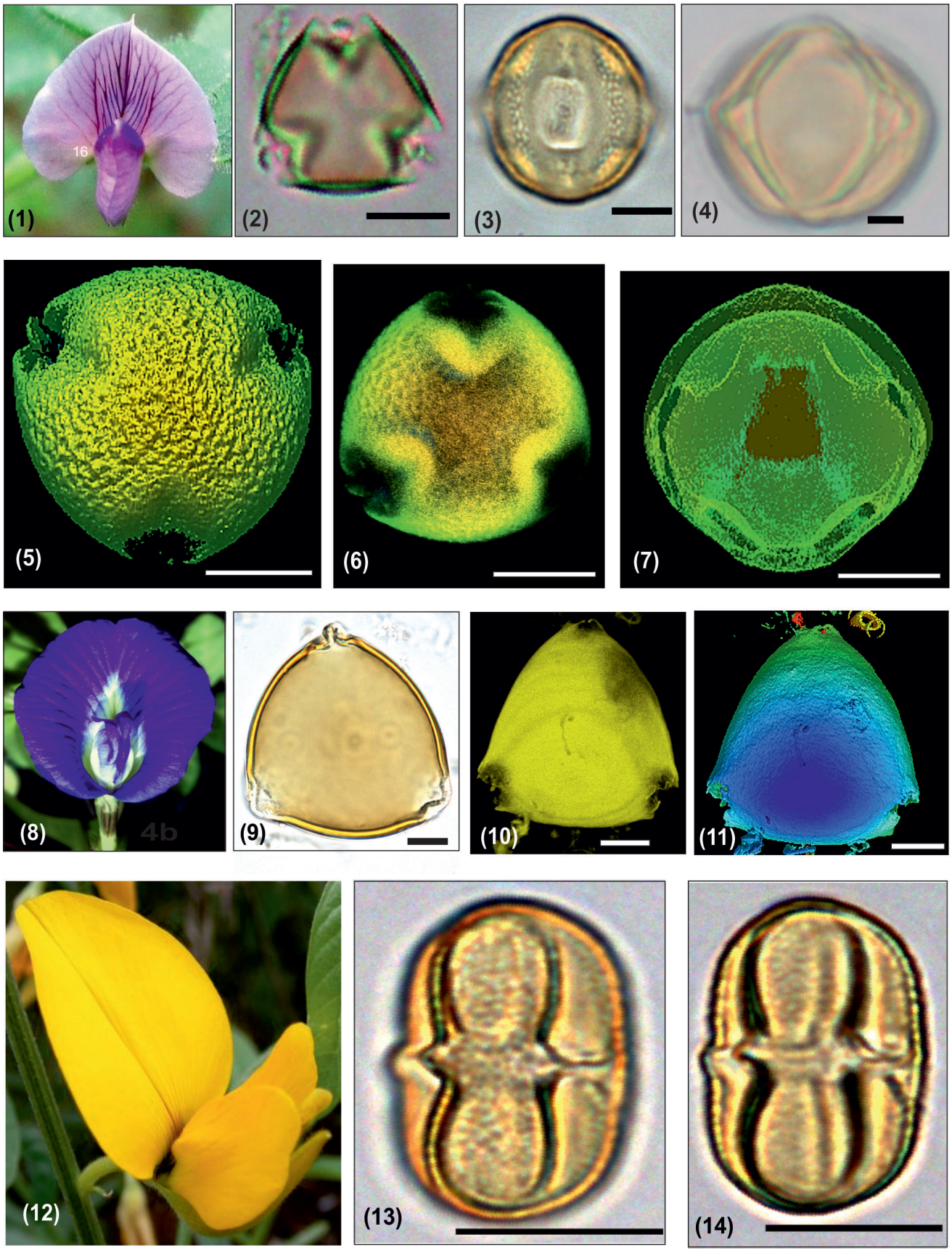

PLATE 2

long, 1.5 (1.67) 2.0 µm wide at equator with acuminate tips, and smooth margins. Ora almost circular, 5.0 (5.33) 6.0 µm in diameter, annulate. Mesocolpia 14.0 (15.0) 16.0 µm, apocolpia 5.0 (5.33) 6.0 µm across. Exine 3.0 µm thick and sexine (2.0 µm) twice as thick as nexine (1.0 µm) [crassisexinous], sculpturing faintly reticulate; tegillate.

Millettia ovalifolia sensu Kurz, Common name: Jewels on a string

(Pl. 5.1–7)

Pollen grains oblate spheroidal to spheroidal, P/E 0.90 (0.95) 1.0, MI, 16.0 (18.0) 20.0 µm x 17.0 (19.0) 20.0 µm, polar outline triangular, equatorial outline circular to elliptical; trizonocolporate. Colpi narrow, linear, 12.0 (14.0) 16.0 µm long, 1.5 (1.83) 2.0 µm wide and protruding at equator, acute tips with smooth margins. Ora lalongate, 4.0 (4.67) 6.0 µm long, 8.0 (9.33) 10.0 µm wide at equator, extended laterally, rectangular. Mesocolpia 8.0 (9.0) 10.0 µm, apocolpia 1.5 (1.75) 2.5 µm across. Exine 2.0 µm thick, sexine (1.0 µm) as thick as nexine (1.0 µm), sculpturing faintly reticulate and tegillate.

Lathyrus oleraceus L.*,* Common name: Field–pea

(Pl. 5.7–14)

Pollen euprolate to perprolate, P/E 1.91 (2.0) 2.1, ME, 42.0 (42.67) 44.0 µm x 20.0 (21.33) 22.0 µm, polar outline subtriangular, equatorial outline elliptical; trizonocolporate. Colpi narrow, linear, 30.0 (32.0) 34.0 µm long, 1.5 (2.0) 2.5 µm wide at equator, tips acuminate, margins smooth. Ora circular to lalongate, 6.0 (6.33) 7.0 µm long, 7.0 (7.33) 8.0 µm wide at equator. Mesocolpia 8.0 (10.0) 12.0 µm, apocolpia 4.0 (5.33) 6.0 µm across. Exine 2.0 µm thick, sexine (1.0 µm) as thick as nexine (1.0 µm) or slightly thicker, sculpturing reticulate, heterobrochate and tegillate.

Pongamia pinnata L., Common Name: Pongam oil tree

(Pl. 6.1–7)

Pollen suboblate, spheroidal to prolate spheroidal, P/E 0.77 (0.92) 1.09, ME, 22.0 (23.5) 24.0 µm x 22.0 (26.0) 31.0 µm, polar outline sub–triangular, equatorial outline elliptical; trizonocolporate. Colpi narrow, linear, 18.0 (19.5) 20.0 µm long, 1.5 (1.8) 2.0 µm wide at equator with acute tips and smooth margins. Ora lalongate, 4.0 (4.25) 4.5 µm long, 5.0 (5.5) 6.0 µm wide at equator. Mesocolpia 16.0 (17.0) 18.0 µm, apocolpia 1.0 (2.0) 3.0 µm across. Exine 1.75–2.0 µm thick, nexine (1.0–1.5 µm) thicker than sexine (0.5–0.75 µm), sculpturing faintly reticulate and tegillate.

Trifolium alexandrinum L., Common name: Egyptian clover

(Pl. 6.7–14)

Pollen grains subprolate to euprolate, P/E 1.31 (1.34) 1.36, ME, 30.0 (31.33) 34.0 µm x 22.0 (23.33) 26.0 µm, polar outline subtriangular, equatorial outline elliptical; trizonocolporate. Colpi narrow, linear, 24.0 (25.33) 28.0 µm long, 2.0 (2.33) 2.5 µm wide at equator with acute tips and smooth margins. Ora lalongate, 4.0 (4.67) 6.0 µm long, 6.0 (7.0) 9.0 µm wide at equator, elliptical. Sexine (1.0 µm) as thick as nexine (1.0 µm), sculpturing reticulate, heterobrochate, tegillate.

Trigonella foenum–graecum L., Common Name: Fenugreek

(Pl. 7.1–6)

Pollen subprolate to euprolate, P/E 1.30 (1.41) 1.56, MI, 22.0 (24.5) 28.0 µm x 16.0 (17.5) 20.0 µm, with triangular polar outline, depressed at three corners, equatorial outline elliptical; trizonocolporate. Colpi narrow, linear, 18.0 (18.5) 20.0 µm long, 1.0 (1.25) 1.5 µm wide at equator with acute tips and smooth margins. Ora circular to lalongate 4.0 (4.5) 6.0 µm long, 6.0 (6.25) 6.5 µm wide at equator, annulate. Mesocolpia 8.0 (10.0) 12.0 µm, apocolpia 2.0 (3.0) 4.0 µm across. Exine 2.0 µm thick, nexine (1.5 µm) much thicker than

PLATE 3

(Scale Bar: LM=20 µm, CLSM=100 µm)

1–3. *Crotalaria juncea* (1–3). CLSM microphotographs, (1). showing depth view with the colpi with endoapertures in different colours, (2). Surface view, (3). Surface ornamentation with deep aperture.

4–10. C*rotalaria retusa* (4). Flower, (5–7). LM microphotographs, (5). General view, (6–7). Equatorial view showing colpi and surface ornamentation (objective 60 x), (8–10). CLSM microphotographs, (8). Equatorial view showing colpi and their position, Surface view showing colpi length and surface ornamentation.

11–15. *Dalbergia sissoo* 11. Flower, (12–13). LM microphotographs, (12). Vertical view showing lalongate Os, (13). Polar view showing wall thickess and aperture position, (14–15). CLSM microphotographs, (14). Equatorial view showing the Os and colpi, (15). Surface view showing ornamentation.

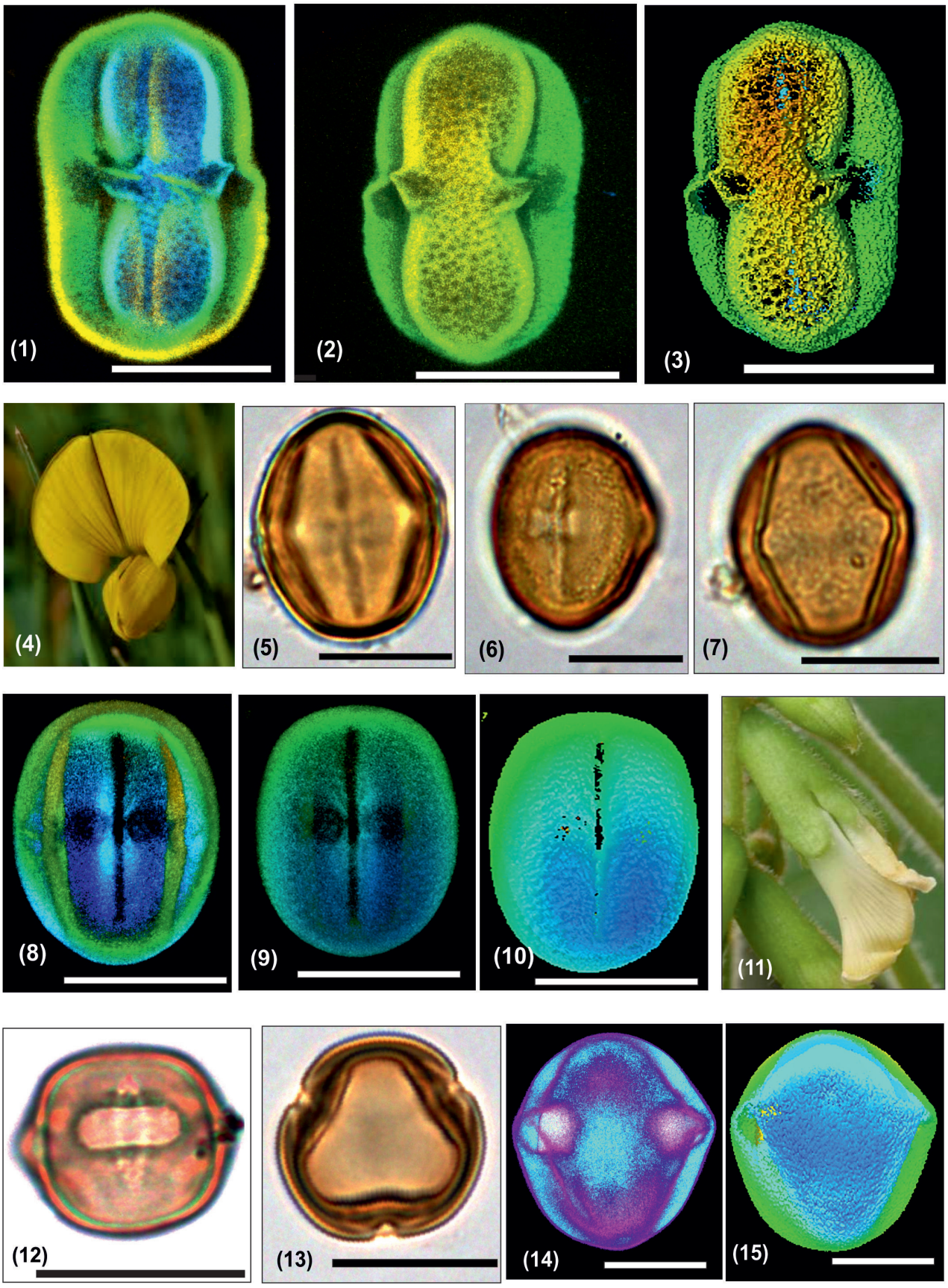

PLATE 3

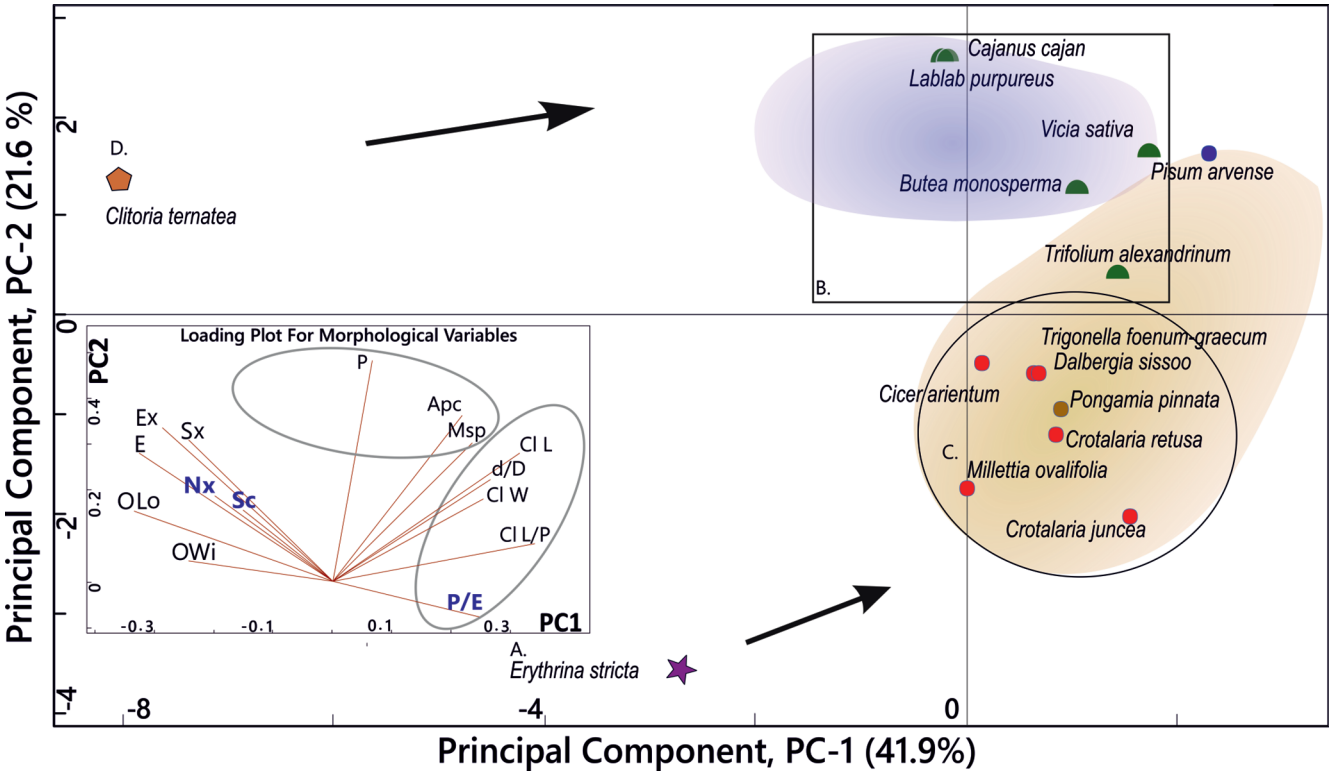

Fig. 2—Principal components analysis score plot expressing the pollen morphological variation of 14 genera and 15 species of Papilionoideae. Morphological characters like P (Polar Axis), E = D (Equatorial Axis), Cl L (Colpi Length), Cl W (Colpi Width), Msp (Mesocolpia), Apc = d (Apocolpia), Ex (Exine), Sx (Sexine), Nx (Nexine), O Lo (Ora Length), O Wi (Ora Width) were studied.

sexine (0.5 µm) [crassinexinous], sculpturing finely reticulate, homobrochate, brochi small and tegillate.

Vicia sativa L., Common name: Common vetch

(Pl. 7.7–14)

Pollen euprolate, P/E 1.42 (1.43) 1.46, ME, 34.0 (35.33) 38.0 µm x 24.0 (24.67) 26.0 µm, polar outline subtriangular, equatorial outline rectangular; trizonocolporate. Colpi narrow and linear, 22.0 (23.33) 24.0 µm long, 1.5 (1.83) 2.0 µm wide at equator with tips acute and smooth margins. Ora almost circular, 4.0 (5.0) 6.0 µm diameter. Mesocolpia 16.0 (17.0) 18.0 µm, apocolpia 5.0 (6.0) 7.0 µm across. Exine 2.0 µm thick, sexine (1.0 µm) as thick as nexine (1.0 µm), and psilate.

DISCUSSIONS

Pollen grains of subfamily Papilionoideae show a wide range of specialized characters including trends in shape from spheroidal to oblate, variation in aperture number and thickness of exine and endexine. Ferguson and Skvarla (1983) identified similar changes in pollen characteristics of Papilionoideae. Based on the different pollen characteristics, the total PCA Score is 73%, whereas PC1 and PC2 variation is 41.9 and 21.6%, respectively (Table 4). Statistical analysis of the palynological evidence reveals close affinities in Colpi

PLATE 4

(Scale Bar: LM=20 µm, CLSM=100 µm)

1–6. *Erythrina stricta* (1). flower, (2–3). LM microphotographs, (2). broad facet, (3). Surface view showing ornamentation, (4–6). CLSM microphotographs, (4–5). Surface view showing ornamentation, (6). Optical view.

7–13. *Lablab purpureus* (7). Flower, (8–10). LM microphotographs, (8). a broad facet of aperture with wall focused, (9). Surface view, Colpi focused, (11–13). CLSM microphotographs, (11). broad facet with aperture focused, (12). Colpi focused, (13). Ornamentation focused.

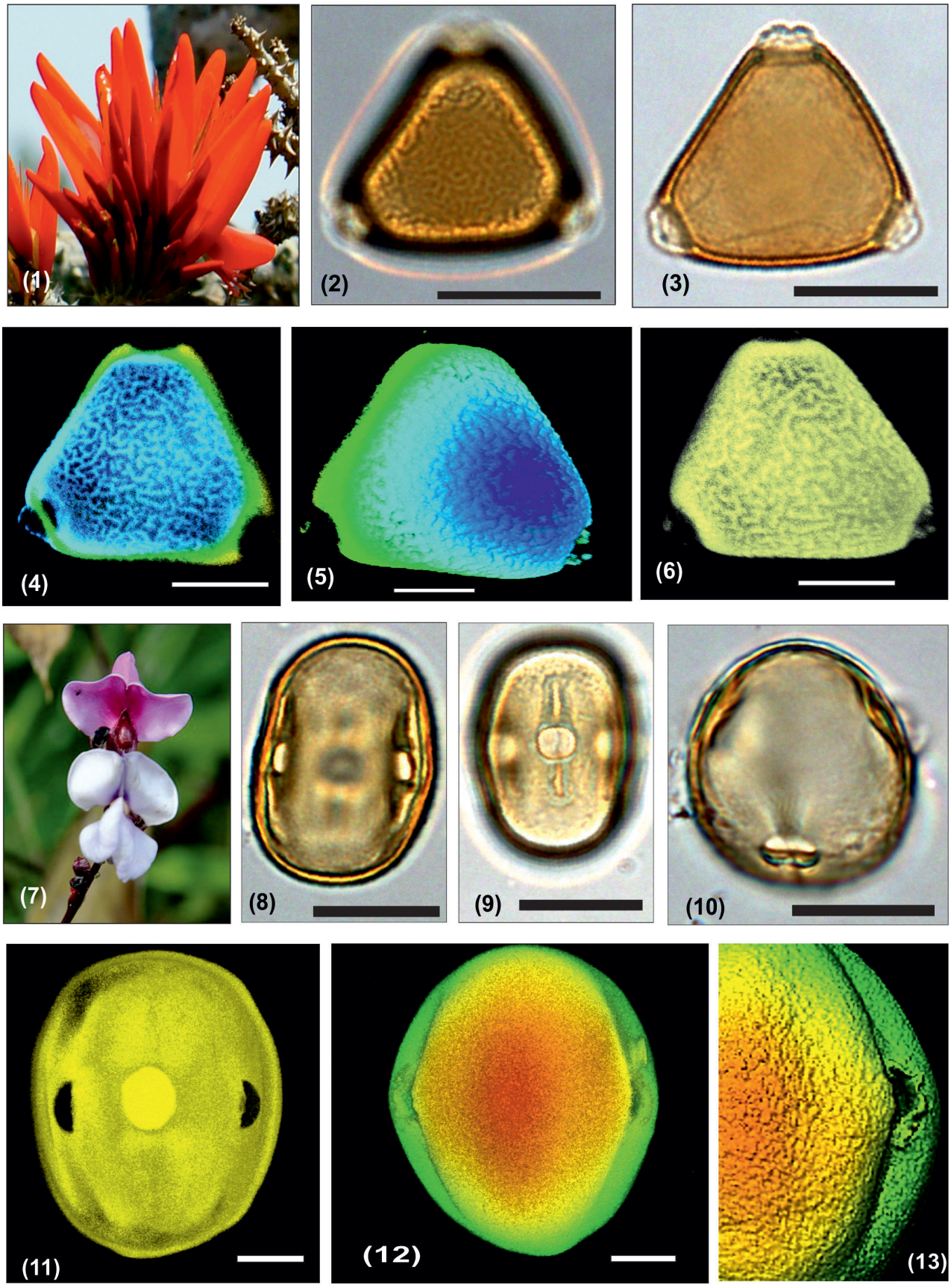

PLATE 4

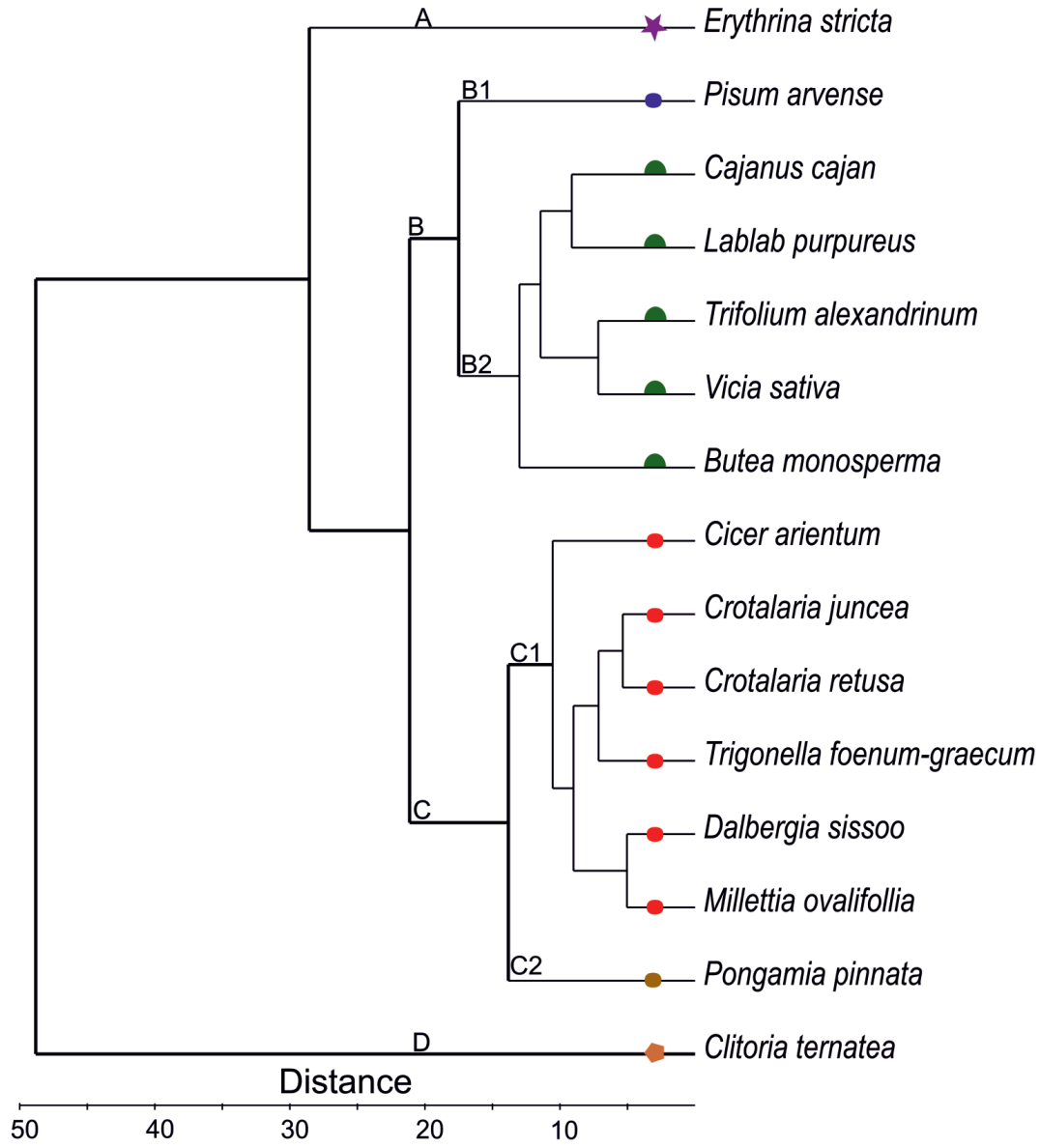

Fig. 3—Phenogram of the 15 species studied by clustering with UPGMA methods.

PLATE 5

(Scale Bar: LM=20 µm, CLSM=100 µm)

1–6. *Millettia ovalifolia* (1). Flower, (2–3). LM microphotographs, (2). General View with wall and apertures focused, (3). An Overview, (4–6). CLSM microphotographs, (4). broad facet with aperture focused, (5). 3D view, (6). Surface ornamentation focused in 3D view.

7–14. *Lathyrus oleraceus L.* (7). Flower (8–10). LM microphotographs, (8). broad facet with ornamentation focussed, (9). broad facet with colpi and apertures focussed, (10). Optical view with wall focussed, (11–14). CLSM microphotographs, (11). 3D view with colpi and surface ornamentation, (12). broad facet with colour depth, (13). Optical view, (14). Ornamentation focused.

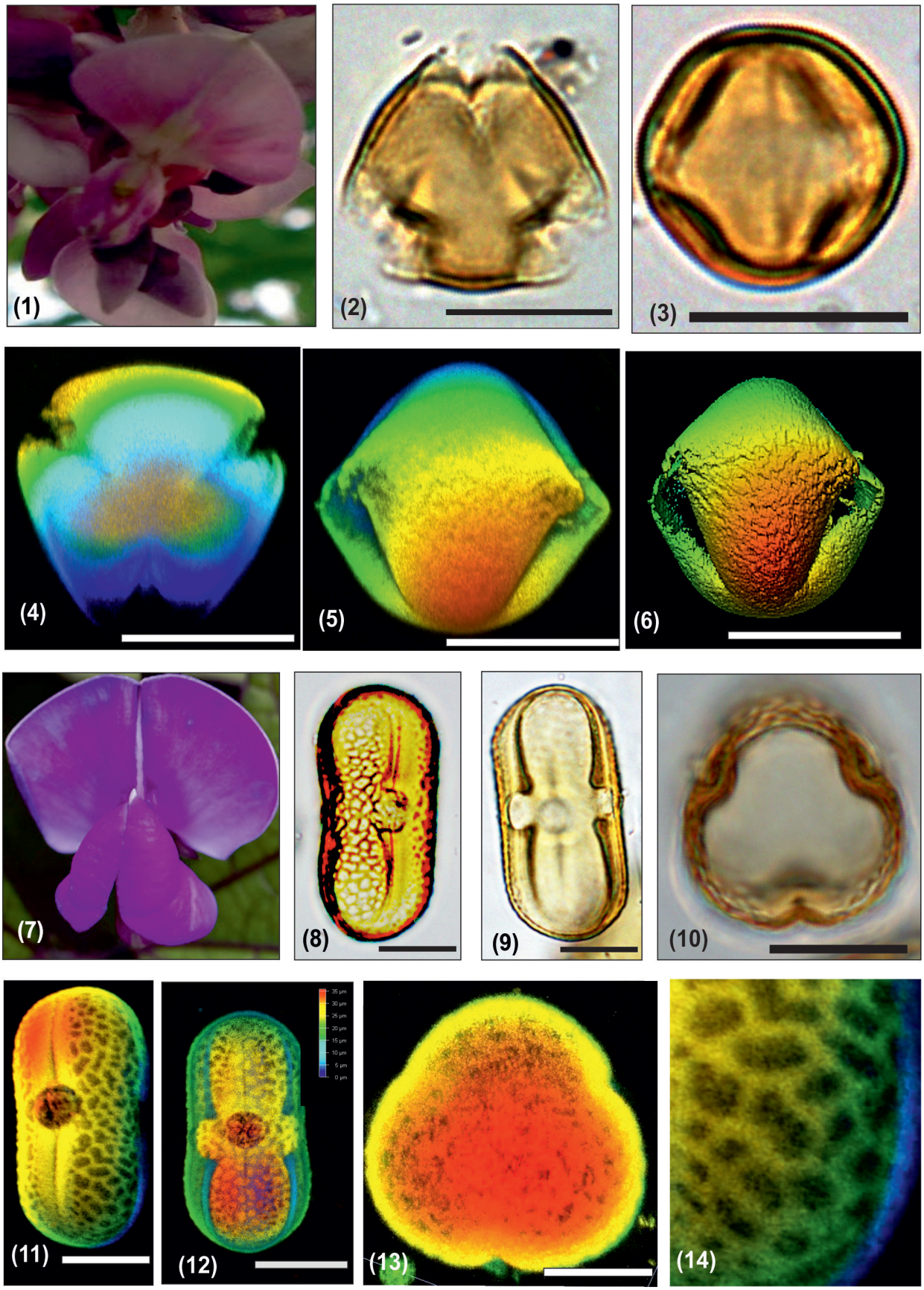

PLATE 5

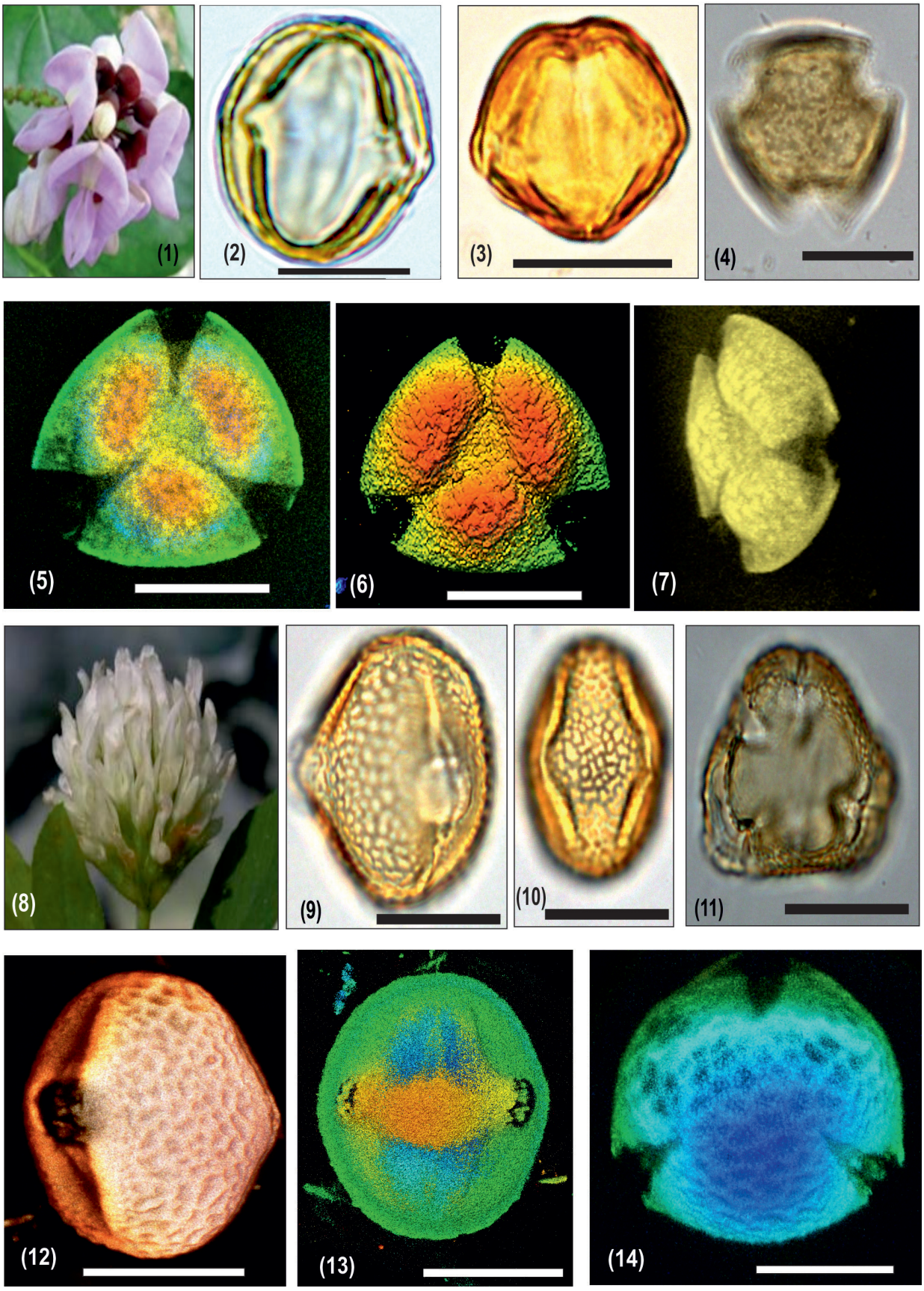

PLATE 6

Length (Cl), Colpi Width, CL/Polar Axis and Apocolpium / Equatorial Axis ratios for PC1 of 10 species (Table 3). Polar Axis, Apocolpium, together with the thickness of Exine and Sexine are prominent characters of affinities for PC2 (3 species) in the Papilionoideae (Table 3).

The attributes of pollen grains of various plant taxa distinguish the plants and categorize them at the generic and/or species level. Hence, detailed pollen morphological characteristics not only aid in distinguishing and categorizing modern vegetation (statistical analysis), but also assist in drawing robust inferences concerning past vegetation/climate change based on the identification of fossil pollen (Selling, 1947).

Comparative pollen morphology research using LM and CLSM data of Papilionoideae, which is known to be eurypalynous (Daluz *et al*., 2013) help in a better understanding of the morphological characteristics of 14 different pollen taxa.

Based on pollen morphometry in LM six types of pollen shape (P/E ratio) have been identified. Type 1 is comprised of *Butea monosperma* (0.94), *Cajanus cajan* (0.92), and *Millettia ovalifolia* (0.95) showing oblate spheroidal to spheroidal shape; Type 2 comprises *Pongamia pinnata* (0.92); *Dalbergia sissoo*, (1.07), *Cicer arietinum* (1.08) and *Lablab purpureus* (1.10) that are suboblate, spheroidal to prolate spheroidal; Type 3 comprises *Clitoria ternatea* (0.48) and *Erythrina stricta* (0.68) that are peroblate to euoblate in shape; Type 4 is euprolate found in *Crotalaria juncea* (1.68) and *Vicia sativa* (1.43); Type 5 is subprolate to euprolate found in *Crotalaria retusa* (1.27), *Trifolium alexandrinum* (1.34) and *Trigonella foenum–graecum* (1.41), Type 6 is euprolate to perprolate found in *Lathyrus oleraceus* (2.0). In general, the pollen with smooth exine and oblong shape are anemophilous and help in dissemination through the wind (Muller, 1979).

On the basis of the CLSM study the exine ornamentation were distinguished into three distinct pollen types, viz. Type 1: psilate *Clitoria ternatea* Type 2: tectate distinctly reticulate (A) Homobrochate (*Butea monosperma* and *Trigonella foenum–graecum*). (B) Heterobrochate *Cajanus cajan, Lathyrus oleraceus Lam., Trifolium alexandrinum* Type 3 tectate faintly reticulate (*Crotalaria* sp.), *Dalbergia sissoo, Millettia ovalifolia, Pongamia pinnata* are distinguished. The data support earlier studies on Trifolieae by Zohary and Heller (1984) and Gazar (2003) and also provides data for future taxonomic and phylogenetic analyses.

Statistical Analysis

The UPGMA phenogram and all OTUs in the survey are presented in Fig. 3. The cophenetic correlation was 92%, indicating a strong percentage of the data similarity matrix is transferred to the phenogram (Fig. 3). Four clusters (A, B, C, D) and sub branches (B1, B2, C1 C2) were formed such as A with a sole representative *Erythrina stricta*, while B is subdivided into sub branch B1 represented by a single pollen taxon *Lathyrus oleraceus* and sub branch B2 with *Cajanus cajan, Lablab purpureus, Trifolium alexandrinum, Vicia sativa, Butea monosperma*. C is a cluster of sub branch C1 with *Cicer arietinum, Crotalaria juncea, Crotalaria retusa*, *Trigonella foenum–graecum, Dalbergia sissoo* and *Millettia ovalifolia* together with sub–branch C2 consisting of *Pongamia pinnata*. Cluster D is only represented by *Clitoria ternatea* in the phenogram.

Principal component analysis (PCA) was undertaken to obtain Eigen analysis results and three dominant principal components of loading scores (Table 1–2, Figs 2–3). In total, three principal components (Fig. 2) describe a 73% correlation of the pollen morphological character variations between 15 species (14 genera) of pollen taxa. The first principal component (PC1) scoring system is dominated by the colpus length (Cl L), colpus width (Cl W), the ratio of the colpus length and polar axis (Cl L/P) and ratios between apocolpium and equatorial axis (d/D). Within the first component a group of 10 species, i.e. *Cicer arietinum*, *Crotalaria juncea*, *Crotalaria retusa*, *Dalbergia sissoo*, *Trigonella foenum–graecum*, *Lathyrus oleraceus*, *Pongamia pinnata*, *Trifolium alexandrinum, Vicia sativa*, and *Millettia ovalifolia*, represents 41.9% of the total variation in morphological characters. The second principal component scoring is dominated by the length of the polar axis (P) and apocolpium (d) along with the thickness of exine (Ex) and sexine (Sx). The second axis group consisting of *Butea monosperma, Cajanus cajan* and *Lablab purpureus* presents 21.6% of the total variation. The third principal component (PC3) scoring system is dominated by the ratio of polar to the equatorial axis (P/E), sculpturing (Sc) and nexine thickness (Nx).

←

PLATE 6

(Scale Bar: LM=20 μm, CLSM=100 μm)

1–7. *Pongamia pinnata* (1). Flower, (2–4). LM microphotographs, (2). Equatorial view with colpi and wall focused, (3). broad facet, (4). Polar view showing ornamentation, (5–7). CLSM microphotographs;

8–14. *Trifolium alexandrinum*, (8). Flower, (9–11). LM microphotographs, (9). An overview, (10). broad facet with colpi and aperture focused, (11). broad facet with ornamentation focused, (12–14). CLSM microphotographs, (12). 3D image with aperture focused, (13). broad facet with aperture focused, (14). broad facet with ornamentation and colour depth.

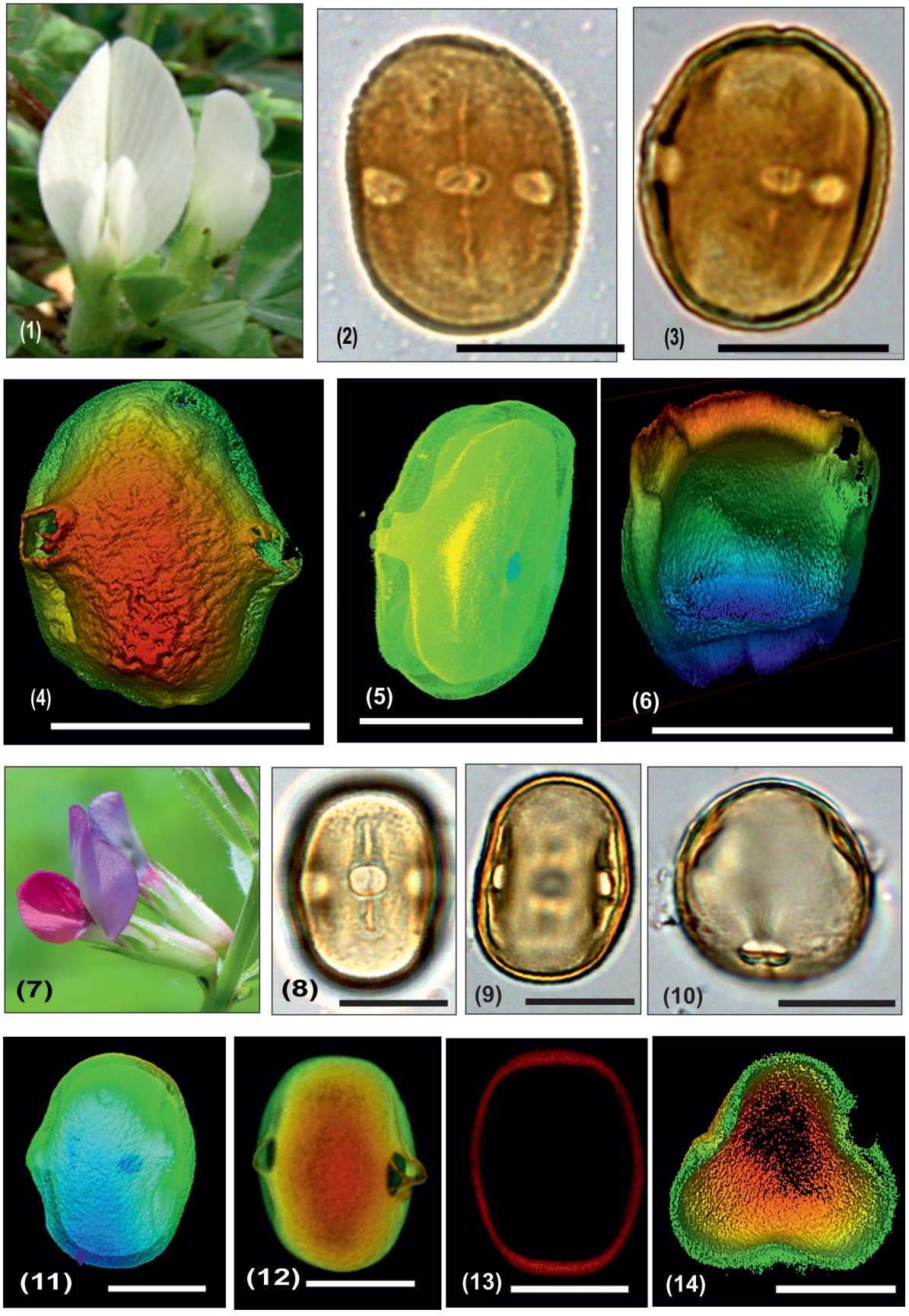

PLATE 7

CONCLUSIONS

1. Majorly, three types of exine ornamentation were distinguished. Type 1: psilate *Clitoria ternatea* Type 2: tectate and distinctly reticulate (A) Homobrochate (*Butea monosperma* and *Trigonella foenum–graecum*). (B) Heterobrochate *Cajanus cajan, Lathyrus oleraceus., Trifolium alexandrinum* Type 3 tectate faintly reticulate (*Crotalaria* sp.), *Dalbergia sissoo, Millettia ovalifolia, Pongamia pinnata*.
2. Both the UPGMA clustering and PCA analysis show that the species *Cicer arietinum*, *Crotalaria juncea*, *Crotalaria retusa*, *Trigonella foenum–graecum*, *Dalbergia sissoo*, and *Millettia ovalfolia* are part of the same cohesive group.
3. *Pongamia pinnata* shares crucial characteristic, such as the dimensions of the colpi and their relation to the polar and the ratio of the apocolpium to the equatorial axis.
4. The apocolpium and polar axis length, as well as the Exine–Sexine thickness, are also similar in length and thickness in *Lathyrus oleraceus*, *Cajanus cajan*, *Lablab purpureus*, *Trifolium alexandrinum*, *Vicia sativa*, and *Butea monosperma*.
5. The study illustrates that the pollen features of all species fully justify their Papilionoideae attribution.

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PLATE 7

(Scale Bar: LM=20 μm, CLSM=100 μm)

1–6. *Trigonella foenum–graecum* (1). Flower, (2–3). LM microphotographs, (2). broad facet with aperture and colpi focused, (3). broad facet with wall and apertures focused, (4–6). CLSM microphotographs, (4). An overview with colour depth, (5). Optical view, (6). Transverse section showing the position of aperture.

7–14. *Vicia sativa* (7). Flower, (8–10). LM microphotographs, (8). broad facet with apertures and wall focused, (9). Surface view, (10). Polar view, (11–14). CLSM microphotographs, (11). Surface ornamentation focused, (12). Surface ornamentation and apertures in 3D view, (13). Optical view, (14). Transverse section showing the holistic view.

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