Pollen morphology of Indigofera (Fabaceae) in China and its taxonomic implications

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Pollen morphology of *Indigofera* (Fabaceae) in China and its taxonomic implications

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Abstract Asia, especially the temperate Sino-Himalayan region is one of the four major diversity centers of *Indigofera*. Pollen morphology of *Indigofera* species in the Sino-Himalayan region is poorly known. In this study, pollen morphology of 52 samples representing 43 Chinese *Indigofera* species and two varieties was examined using scanning electron microscopy to evaluate its taxonomic significance. Parasyncolpate pollen grains were described in *Indigofera* for the first time. Cluster and principal component analyses were conducted based on four quantitative and three qualitative pollen characters. Five groups were recognized within Chinese *Indigofera* in the cluster analysis, but only one can be separated by the first three principal components. The shape and size of pollen grains in *Indigofera* are highly variable. Consequently, both the characters lack any significant taxonomic value. The number of apertures, tectum architecture and perforation density shows some taxonomic significance. The pollen morphology exhibits obvious phylogenetic and biogeographical significance on large scales in *Indigofera*. Although pollen characters alone are insufficient to reconstruct the taxonomic relationships within *Indigofera*, palynological data can provide some useful information for the species-level revisions.

Keywords Fabaceae · *Indigofera* · Palynology · Scanning electron microscopy · Taxonomy

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Introduction

Indigofera L. is the third largest genus in Fabaceae, comprising approximately 750 species (Schrire et al. 2005, 2009). The genus has a pantropical distribution with four major diversity centers: Africa and Madagascar (ca. 550 spp.), Asia, especially the temperate Sino-Himalayan region (ca. 105 spp.), Australia (ca. 50 spp.), and the New World (ca. 45 spp.) (Schrire et al. 2009). *Indigofera* species are annuals or perennials, and habit varies from prostrate and erect herbs to undershrubs, robust shrubs, and rarely trees.

Infrageneric classification of *Indigofera* has long been controversial. At least twelve revisions (Schrire 1995) have been proposed based on morphological characters since the establishment of the genus in 1753 (Linnaeus 1753). The most recent and comprehensive classification was proposed by Schrire (1995) based on a morphological cladistic analysis of a dataset containing 58 morphological characters of ca. 600 Indigofereae species, in which 25 sections and 14 groups were recognized within *Indigofera*. Subsequently, combined DNA (nrITS or plastid DNA) sequences and morphological data analyses revealed that *Indigofera* species clustered into four monophyletic clades, i.e., Palaeotropical clade, Pantropical clade, Cape clade and Tethyan (Boreotropical) clade (Barker et al. 2000; Schrire et al. 2003, 2009).

In China, Fang and Zheng (1989, 1994) recognized 80 species and eight varieties, and they also divided Chinese *Indigofera* species into three subgenera and 14 subsections chiefly based on the shape of fruits, the number of leaflets, and the types of trichomes. In a recent revision of Chinese *Indigofera*, Gao and Schrire (2010) recognized 79 species and nine varieties, of which 45 are endemic. The infrageneric classification of Fang and Zheng (1989, 1994) was not adopted by Gao and Schrire (2010), and no new infrageneric classification was reconstructed. Recently, *I. pseudonigrescens* X.F.Gao & Xue Li Zhao (2015), a new species from China, was published. Only a few Chinese *Indigofera* species have been involved in previous studies (Barker et al. 2000; Schrire et al. 2003, 2009), and the systematics of Chinese *Indigofera* species remains poorly understood.

Pollen morphology of *Indigofera* was examined between 1980 and 2000 (Ferguson and Strachan 1982; Wu and Huang 1995; Schrire and Sims 1997), mainly for African species. Using a sampling of 77 *Indigofera* species, Ferguson and Strachan (1982) defined four types and eight subtypes of pollen morphology based on the pollen size, shape, tectal sculpturing and exine stratification. Wu and Huang (1995) proposed a different classification of pollen for Taiwan *Indigofera* species, and these authors recognized five types of pollen morphology characterized by exine structure, perforations, shape of tectum channels, granular or columellate interstitium, and prominent or reduced foot layer. Schrire and Sims (1997) examined 79 *Indigofera* species including two species of *Vaughania* S.Moore that has been reduced to synonymy under *Indigofera* (Schrire 2008). In the study (Schrire and Sims 1997), perforation density on pollen grain surfaces was newly added, and it was also revealed to be of significant taxonomic value. Cluster analysis recognized three major pollen groups in *Indigofera*.

Previous studies have provided extensive palynological data in *Indigofera* (Ferguson and Strachan 1982; Wu and Huang 1995; Schrire and Sims 1997), but pollen morphology of Chinese endemisms is still largely unknown. Thus far, only two Chinese endemisms (*I. dumetorum* Craib and *I. taiwaniana* T.C.Huang & M.J.Wu) have been studied (Ferguson and Strachan 1982; Wu and Huang 1995). In the present study, we aim to (1) investigate the pollen morphology of Chinese *Indigofera* species using scanning electron microscopy (SEM), and (2) assess the potential significance of pollen characters in the taxonomy of *Indigofera*.

Materials and methods

Sampling and scanning electron microscopy

Pollen grains examined in this study were obtained from the opened flowers, in which the anthers were still wrapped by the keel. Most of the flowers were removed from herbarium specimens deposited in Herbarium of Chengdu Institute of Biology (CDBI), Chinese Academy of Sciences (CAS), and some were obtained from the transplanted plants. In total, 52 samples representing 43 species and two varieties were examined, of which 29 species and one variety are endemic to China. For species with a large geographic distribution, at least two samples from different geographic areas were examined. A list of taxa sampled with voucher information is shown in "Appendix" section.

Pollen grains were directly taped on stubs and sputter coated with gold–palladium. Observations were conducted using scanning electron microscope (Phenom proX, Phenom-world, The Netherlands) at 10 kV at Chengdu Institute of Biology, CAS. SEM photographs of the polar and equatorial views were taken at 7 or 8 K, and the exine surfaces of the apocolpium and mesocolpium areas were both taken at 25 K.

Pollen character

Pollen measurements were performed on digital SEM images using software ImageJ (Abràmoff et al. 2004). For each sample, the polar length (*P*), equatorial length (*E*), the length of colpi (*C*), and the diameter of perforations were measured for at least 20 pollen grains. The ratio of polar length to equatorial length (*P*/*E*) and that of colpus length to polar length (*C*/*P*) were calculated. Descriptive terminology follows that of Erdtman (1952), Ferguson and Strachan (1982), and Punt et al. (2007). Perforation density on pollen grain surfaces was calculated according to the method described by Schrire and Sims (1997). The number of perforations in 1 cm^2 area in the same apocolpium or mesocolpium areas of each pollen grain was counted and converted to number per μm^2, and then used in the analyses.

Phenetic analysis

Cluster analysis (CA) and principal component analysis (PCA) were performed using the program SPSS version 21 (IBM SPSS Statistics). Prior to doing the CA and PCA, the data were standardized. The characters used in the CA and PCA included four quantitative and three qualitative characters: *P*, *P*/*E*, *C*/*P*, the number of apertures, tectum architecture in the apocolpium and mesocolpium areas, and perforation density in the apocolpium area. The three qualitative characters were coded. Tectum architecture in the apocolpium area was treated as five types: (1) imperforate, (2) perforate, (3) microperforate, (4) finely microperforate, and (5) pitted perforate. Tectum architecture in the mesocolpium area was classified into six types: (1) perforate, (2) microperforate, (3) finely microperforate, (4) rugulate and perforate, (5) pitted microperforate, and (6) pitted perforate. The number of apertures was treated as two types: (1) three apertures, and (2) three and more than three apertures.

Hierarchical cluster analysis was conducted using the shortest Euclidean distances according to the Ward's (1963) method. For principal component analysis, the standardized data were computed to obtain the correlation matrix. The first three principal components representing the maximum variance were extracted and plotted as a scatter diagram (Fig. 4).

Results

Pollen morphology

Pollen characters of all examined samples are summarized in Table 1. Representative pollen grains are illustrated in Figs. 1 and 2, while the others are illustrated as in the electronic supplementary material. Pollen grains are shed as monads, and their sizes range from small to medium. The average polar length is 18.86 µm (*I. pseudonigrescens*; Fig. 2i) −28.63 µm (*I. hirsuta* L., Fig. 2h), and the average equatorial length is 23.41 µm (*I. suffruticosa* Mill.; VI: f) −33.49 µm (*I. hirsuta* L., Fig. 2h) µm. *Indigofera hirsuta* possesses the largest ($P \times E = 28.83$ µm × 33.49 µm) pollen grains, while *I. pseudonigrescens* has the smallest ($P \times E = 18.86$ µm × 24.08 µm). Three-colporate and four-colporate pollen grains were observed in all and eight species (i.e., *I. carlesii* Craib, *I. chaetodonta* Franch., *I. dumetorum*, *I. henryi* Craib, *I. heterantha* Wall., *I. jikongensis* Y.Y.Fang & C.Z.Zheng, *I. pampaniniana* Craib and *I. pseudonigrescens*), respectively. We also identified parasyncolpate pollen grains in three species and one variety (i.e., *I. carlesii*, *I. chaetodonta*, *I. decora* var. *ichangensis* (Craib) Y.Y.Fang & C.Z.Zheng and *I. suffruticosa*) (Figs. 1, 2 and I–VII). The shapes of the 3-colporate pollen grains are spherical to oblate, with the ratio of polar axis to equatorial axis varying from 0.71 (*I. bungeana* Walp. 4; II: b and *I. silvestrii* Pamp. 1; VI: b) to 0.92 (*I. megaphylla* X.F.Gao; V: a). The average length of colpi ranges from 13.29 µm (*I. bungeana* 3; II: a) to 21.28 µm (*I. pendula* Franch.; V: d). The ratio of the colpus length to polar length is from 0.52 (*I. hirsuta*; Fig. 2h) to 0.91 (*I. franchetii* X.F.Gao & Schrire; III: d).

Results of CA and PCA

Five groups (Fig. 3a–e) were resolved in cluster analysis at the Euclidean distance of 7.5. Pollen of group A can be distinguished by a combination of 3-colporate pollen grains and microperforate tectum. Group B is unique in having pollen with more than three apertures. Group C pollen is mainly distinguished by the low perforate density in the apocolpium area, while group D differs mainly by the pitted perforate/microperforate. Pollen of group E is characterized by the finely microperforate tectum. The scatter diagram of the first three principal components from the PCA is shown in Fig. 4. The first three principal components accounted for the total variance of 70.27 % in differentiating the taxa. The first principal component explained 32.63 % of the total variance, the second principal component accounted for 21.99 %, while the third principal component accounted for 15.65 %. Two quantitative characters (*P* and *C*/*P*) and one qualitative character (tectum architecture in the mesocolpium area) in the first axis, tectum architecture and perforation density in the apocolpium area in the second axis, and the number of apertures in the third axis showed the strongest loadings. The three-dimensional scatter plot resulting from PCA indicated that all the samples analyzed can be divided into two groups. Group E can be recognized as an independent group, while overlap occurs among groups A, B, C and D.

Discussion

Pollen shape and size

Pollen shape and size have been considered as having a significant taxonomic value in *Indigofera* (Ferguson and Strachan 1982; Schrire and Sims 1997). In this study, the stability of pollen shape and size at infraspecific level was tested among the multiple samples of *I. amblyantha* Craib, *I. bungeana* and *I. silvestrii*. The results indicated that pollen shape and size are relatively stable at the infraspecific level (Table 1). However, pollen shapes and sizes showed dramatic differences when comparing our results with those from previous studies (Ferguson and Strachan 1982; Wu and Huang 1995; Schrire and Sims 1997). For example, the ratios of *P*/*E* for *I. colutea* (Burm.f.) Merr., *I. decora* Lindl., *I. hirsuta*, *I. dumetorum*, *I. linnaei* Ali, *I. pendula*, *I. stachyodes* Lindl. and *I. suffruticosa* were reported to be 0.91, 1.14, 1.13, 1.11, 1.03, 1.12, 1.19 and 0.94 in previous studies as opposed to 0.77, 0.79, 0.85, 0.79, 0.76, 0.89, 0.87 and 0.86 reported in this study, respectively. According to the results of our preliminary

Table 1 Summary of pollen details in *Indigofera* examined in this study

Species	Polar length (*P*) µm	Equatorial length (*E*) µm	*P*/*E*	No. of apertures	Colpus length (*C*) µm	*C*/*P*	Tectum architecture		Avg. perforation (µm^2)	
							Apocolpium	Mesocolpium	Apocolpium	Mesocolpium
I. amblyantha 1	20.17 (18.92–21.33)	25.86 (23.73–27.87)	0.78	3	16.13 (15.31–16.73)	0.8	m. perf.	m. perf.	19.3	22.7
I. amblyantha 2	21.42 (19.64–22.76)	24.50 (21.68–25.64)	0.87	3	17.61 (16.97–18.65)	0.82	m. perf.	m. perf.	16.7	21.3
I. amblyantha 3	23.30 (21.69–24.24)	27.41 (25.04–28.70)	0.85	3	17.25 (16.77–18.69)	0.74	m. perf.	m. perf.	6.7	21.3
I. argutidens	22.81 (20.61–24.14)	26.17 (24.99–29.39)	0.87	3	17.46 (16.76–19.23)	0.76	m. perf.	m. perf.	11.3	42.7
I. atropurpurea	22.28 (20.98–23.42)	26.48 (24.22–28.28)	0.84	3	18.24 (16.38–19.06)	0.82	perf.	perf.	5.3	13.3
I. balfouriana	21.17 (19.66–24.06)	27.14 (25.60–28.85)	0.78	3	17.68 (17.45–17.89)	0.84	m. perf.	m. perf.	13.3	17.3
I. bracteata	27.82 (26.97–28.91)	29.82 (28.53–31.14)	0.93	3	17.27 (16.13–18.03)	0.62	m. perf.	perf.	0	30.7
I. bungeana 1	22.00 (20.17–22.95)	26.92 (25.48–28.43)	0.82	3	16.62 (15.09–17.69)	0.76	m. perf.	m. perf.	12.7	18.7
I. bungeana 2	21.25 (19.28–22.19)	25.57 (23.63–26.29)	0.83	3	17.49 (15.61–19.71)	0.82	m. perf.	m. perf.	18.7	30.7
I. bungeana 3	18.93 (16.94–19.70)	24.20 (20.97–25.84)	0.77	3	13.29 (11.83–15.99)	0.71	m. perf.	m. perf.	20.7	29.3
I. bungeana 4	20.13 (17.94–21.56)	26.39 (25.60–28.12)	0.71	3	14.21 (13.02–16.36)	0.71	m. perf.	m. perf.	10.7	14.7
I. calcicola	25.22 (23.30–26.52)	27.95 (26.25–29.29)	0.9	3	19.89 (19.36–20.88)	0.79	perf.	perf.	22.7	21.3
I. carlesii	24.21 (21.13–28.25)	29.11 (25.70–30.63)	0.83	3,4,5/6	17.41 (15.53–20.73)	0.72	m. perf.	p. m. perf.	14.7	18.7
I. cassioides	24.17 (22.83–24.65)	28.45 (27.27–29.42)	0.85	3	18.98 (18.53–19.74)	0.79	m. perf.	m. perf.	13.3	18.7
I. chaetodonta 1	21.30 (18.73–23.41)	25.10 (22.21–27.12)	0.85	3	16.09 (14.67–17.98)	0.76	perf.	perf.	21.3	17.3
I. chaetodonta 2	–	–	–	3, 4,4–12	–	–	perf.	perf.	24	28
I. colutea	21.46 (19.91–23.74)	27.80 (24.77–30.84)	0.77	3	15.86 (14.95–17.73)	0.65	f. m. perf.	f. m. perf.	69.3	73.3
I. decora	25.38 (22.15–29.07)	29.47 (27.76–30.63)	0.86	3	17.34 (15.71–19.46)	0.68	m. perf.	p. m. perf.	2.7	12.7
I. decora var. *ichangensis*	24.16 (20.93–25.62)	30.13 (28.87–32.03)	0.80	3, 5/6	19.54 (18.75–20.11)	0.78	m. perf.	p. m. perf.	5.3	30.7
I. delavayi	25.14 (24.64–25.50)	32.53 (31.75–34.33)	0.77	3	17.10 (15.23–18.41)	0.71	perf.	p. perf.	0	10.7
I. dolichochaete	21.02 (19.18–22.85)	24.38 (23.37–25.36)	0.86	3	18.67 (18.00–19.60)	0.89	m. perf.	m. perf.	13.3	21.3
I. dumetorum	24.37 (22.84–26.17)	30.76 (29.18–33.43)	0.79	3, 4	18.63 (18.51–18.77)	0.76	p. perf.	p. perf.	14.7	11.3
I. esquirolii	24.53 (22.25–25.99)	26.93 (25.66–28.62)	0.91	3	17.05 (15.45–17.99)	0.7	p. perf.	p. perf.	0	10.7
I. franchetii	22.00 (20.50–24.45)	26.17 (25.56–27.12)	0.84	3	19.96 (17.89–22.30)	0.91	m. perf.	m. perf.	10.7	25.3
I. hancockii	21.56 (20.64–22.13)	27.81 (26.41–28.70)	0.78	3	18.35 (16.94–19.37)	0.85	perf.	perf.	21.3	25.3
I. hebepetala var. *glabra*	28.45 (27.27–30.36)	31.40 (30.43–32.67)	0.91	3	15.67 (14.23–16.70)	0.55	perf.	perf.	0	14.7
I. hendecaphylla	21.22 (20.49–22.86)	28.59 (27.19–29.81)	0.74	3	16.69 (16.32–17.10)	0.79	f. m. perf.	f. m. perf.	70.7	72
I. henryi	23.23 (21.99–25.61)	25.03 (23.63–26.48)	0.93	3, 4	16.63 (15.04–18.10)	0.72	m. perf.	m. perf.	22.7	21.3
I. heterantha	22.21 (21.72–23.24)	29.85 (28.76–31.50)	0.74	3, 4	17.18 (15.81–18.86)	0.77	perf.	perf.	10.7	48
I. hirsuta	28.63 (25.41–30.52)	33.49 (29.73–36.11)	0.85	3	14.86 (11.37–17.52)	0.52	perf.	r. and perf.	6.7	9.3
I. jikongensis	25.10 (27.23–23.84)	31.65 (28.62–34.90)	0.79	3, 4	17.61 (15.76–19.97)	0.7	m. perf.	p. m. perf.	0	21.3
I. lenticellata	24.43 (22.08–25.57)	27.16 (25.36–28.79)	0.9	3	17.92 (15.86–18.85)	0.73	perf.	perf.	10.7	14.7
I. linnaei	22.04 (19.30–24.92)	29.03 (26.25–31.28)	0.76	3	18.20 (17.14–19.12)	0.83	m. perf.	f. m. perf.	17.3	17.3

Table 1 continued

Species	Polar length (P) µm	Equatorial length (E) µm	P/E	No. of apertures	Colpus length (C) µm	C/P	Tectum architecture		Avg. perforation (µm^2)	
							Apocolpium	Mesocolpium	Apocolpium	Mesocolpium
I. mairei	21.83 (20.02–23.31)	27.13 (26.39–28.12)	0.8	3	17.12 (16.38–18.64)	0.78	m. perf.	m. perf.	18.7	18.7
I. megaphylla	22.60 (21.78–23.35)	24.52 (21.31–26.16)	0.92	3	16.76 (16.24–17.52)	0.74	m. perf.	m. perf.	13.3	21.3
I. pampaniniana	24.29 (22.71–25.99)	28.96 (25.33–31.50)	0.84	3, 4	18.00 (16.89–20.09)	0.74	m. perf.	m. perf.	6.7	22.7
I. parkesii	22.24 (20.53–23.62)	30.44 (27.77–34.23)	0.73	3	18.88 (18.24–19.21)	0.85	m. perf.	p. m. perf.	22.7	20
I. pendula	27.58 (26.21–29.00)	31.11 (28.10–34.20)	0.89	3	21.28 (20.79–21.70)	0.77	m. perf.	m. perf.	13.3	22.7
I. penduloides	23.49 (21.82–25.16)	30.90 (29.33–33.09)	0.76	3	17.02 (14.47–19.80)	0.72	m. perf.	m. perf.	14.7	24
I. pseudoheterantha	25.26 (21.99–27.64)	33.29 (31.74–34.66)	0.76	3	14.66 (13.79–15.45)	0.58	m. perf.	r. and perf.	6.7	12
I. pseudonigrescens	18.86 (18.16–19.92)	24.08 (23.48–25.12)	0.78	3, 4	13.51 (13.04–14.01)	0.72	m. perf.	m. perf.	29.3	33.3
I. rigioclada	20.18 (19.02–20.98)	26.10 (24.58–27.20)	0.77	3	14.45 (13.80–14.91)	0.72	m. perf.	m. perf.	18.7	16
I. scabrida	21.86 (20.88–23.54)	26.49 (23.51–28.91)	0.83	3	16.16 (15.82–16.64)	0.74	m. perf.	p. m. perf.	0	21.3
I. sensitiva	20.61 (19.45–21.56)	24.76 (23.93–25.51)	0.83	3	15.01 (14.45–15.32)	0.73	perf.	perf.	26.7	18.7
I. silvestrii 1	19.24 (17.70–20.38)	27.24 (26.22–28.02)	0.71	3	15.77 (14.37–16.75)	0.82	perf.	perf.	25.3	24
I. silvestrii 2	20.98 (20.04–22.64)	24.51 (23.68–25.29)	0.86	3	15.40 (15.11–15.07)	0.73	m. perf.	perf.	30.7	30.7
I. stachyodes	22.97 (21.26–24.55)	26.41 (24.62–29.76)	0.87	3	16.49 (15.24–18.75)	0.72	m. perf.	p. perf.	1.3	10.7
I. sticta	21.17 (20.44–21.77)	23.55 (23.16–24.10)	0.9	3	15.53 (14.56–17.06)	0.73	m. perf.	m. perf.	33.3	30.7
I. suffruticosa	20.20 (18.40–21.68)	23.41 (21.42–25.30)	0.86	3, 5–12	16.03 (14.63–17.17)	0.79	m. perf.	m. perf.	21.3	28
I. szechuensis	23.60 (22.23–25.06)	30.50 (29.48–32.87)	0.77	3	19.19 (18.47–19.98)	0.81	m. perf.	m. perf.	26.7	16
I. wightii	24.63 (23.07–25.45)	30.21 (28.63–32.63)	0.82	3	16.47 (14.26–17.83)	0.67	f. m. perf.	f. m. perf.	86.7	96
I. wilsonii	25.19 (21.82–28.28)	29.41 (27.87–31.58)	0.86	3	17.67 (15.97–20.97)	0.7	p. perf.	p. m. perf.	10.7	17.3

A dash (–) indicates missing data

perf. perforate, *m. perf.* microperforate, *f. m. perf.* finely microperforate, *p. perf.* pitted perforate, *r.* rugulate

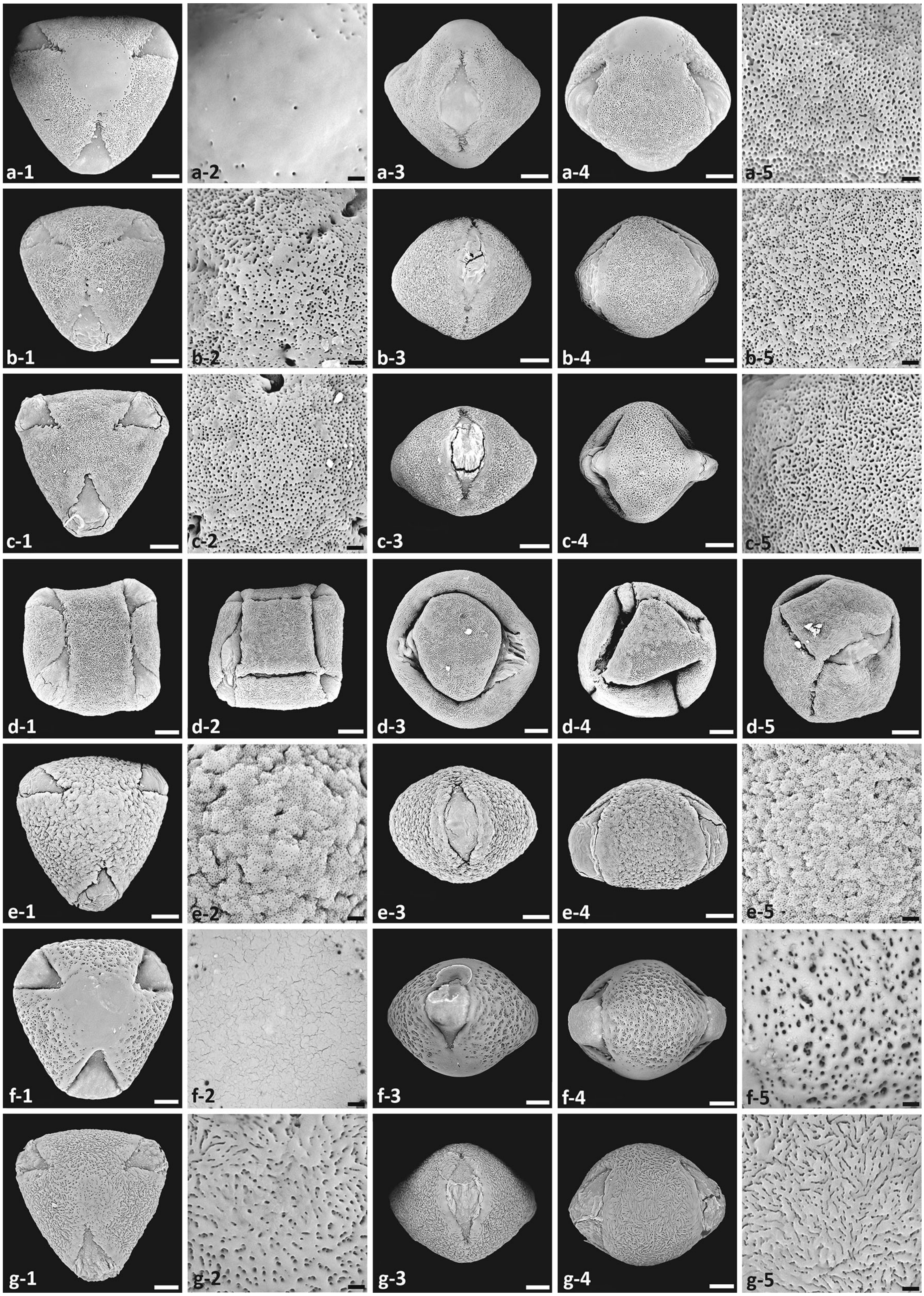

Fig. 1 SEM micrographs of pollen grains in *Indigofera*. **a-1–c-1**, **e-1–g-1** Pollen grains in polar view, *scale bar* 5 μm; **a-2–c-2**, **e-2–g-2** Exine surfaces in polar view, *scale bar* 0.5 μm; **a-3–c-3**, **e-3–g-3** Apertures of pollen grains in equatorial view, *scale bar* 5 μm; **a-4–c-4**, **e-4–g-4** Pollen grains in equatorial view, *scale bar* 5 μm; **a-5–c-5**, **e-5–g-5** Exine surfaces in equatorial view, *scale bar* 0.5 μm; **d**:**1–5** Parasyncolpate pollen grains, *scale bar* 5 μm. **a** *I. bracteata*. **b** *I. bungeana* 2. **c** *I. chaetodonta* 1. **d** *I. chaetodonta* 2. **e** *I. colutea*. **f** *I. delavayi*. **g** *I. dumetorum*

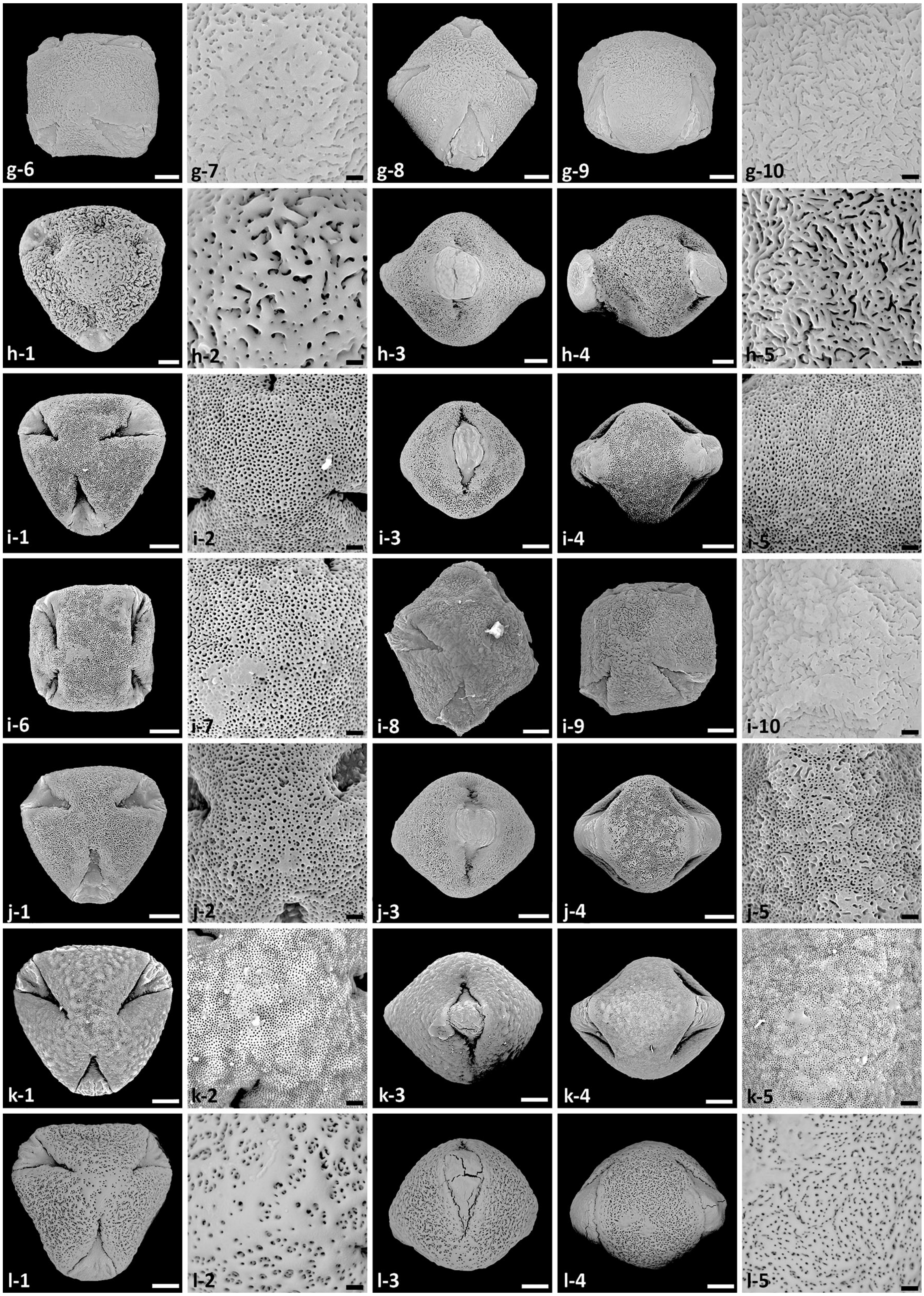

Fig. 2 SEM micrographs of pollen grains in *Indigofera*. **g-6**, **h-1**, **i-1**, **i-6**, **j-1**–**l-1**. Pollen grains in polar view, *scale bar* 5 µm; **g-7**, **h-2**, **i-2**, **i-7**, **j-2**–**l-2**. Pollen grains in equatorial view, *scale bar* 0.5 µm; **g-8**, **h-3**, **i-3**, **i-8**, **j-3**–**l-3** Apertures of pollen grains in equatorial view, *scale bar* 5 µm; **g-9**, **h-4**, **i-4**, **i-9**, **j-4**–**l-4** Pollen grains in equatorial view, *scale bar* 5 µm; **g-10**, **h-5**, **i-5**, **i-10**, **j-5**–**l-5** Exine surfaces in equatorial view, *scale bar* 0.5 µm. **g** *I. dumetorum*. **h** *I. hirsuta*. **i** *I. pseudonigrescens*. **j** *I. sensitiva*. **k** *I. wightii*. **l** *I. wilsonii*

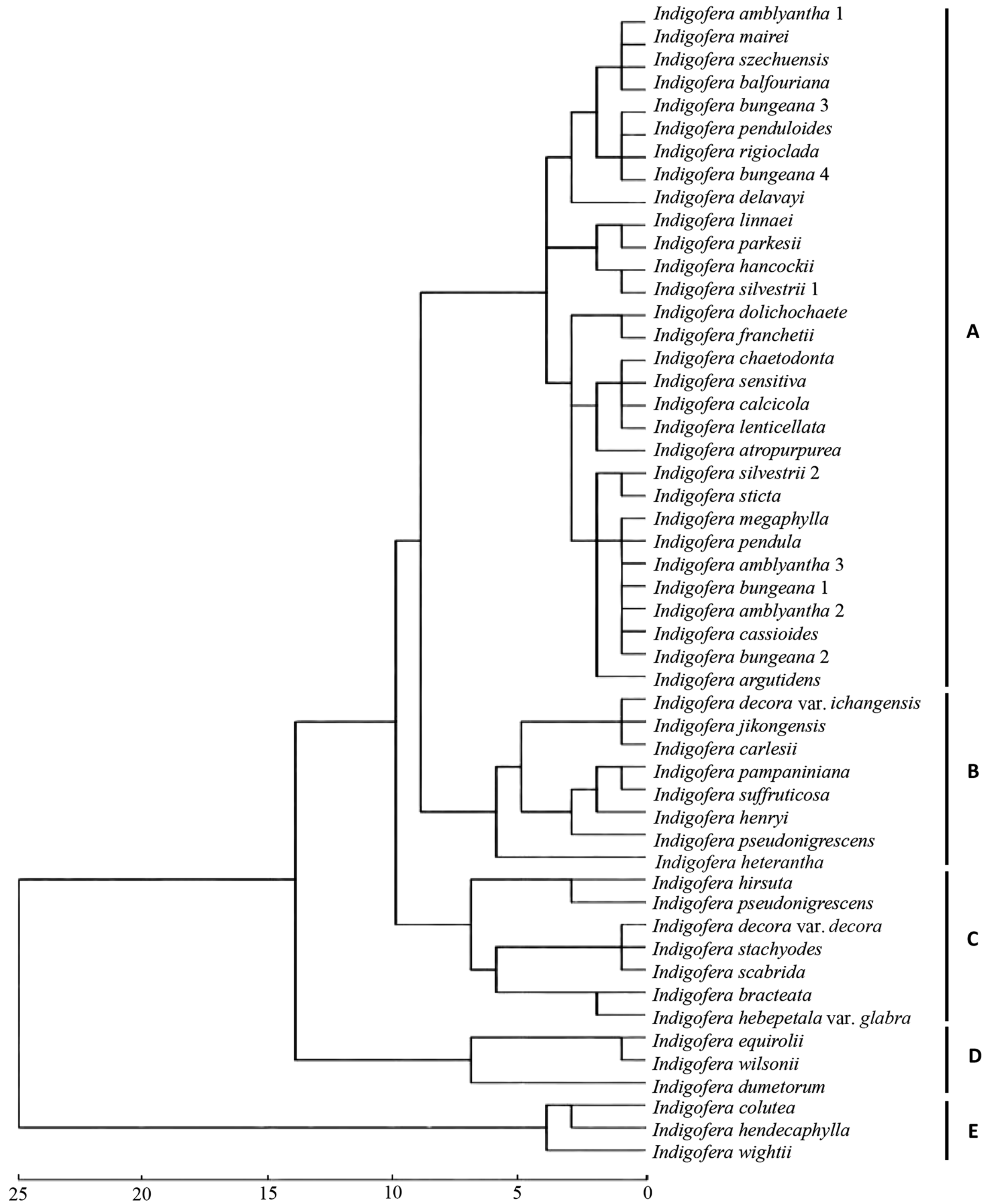

Fig. 3 Phenogram of the cluster analysis (CA)

investigations (see Online Resource 2), these differences may result from the differences in material selections. As a consequence, material selection is critical for palynological study of *Indigofera*. The pollen shape and size are not suitable to be treated as valuable taxonomic characters in *Indigofera*.

Number of apertures

The number of apertures was generally reported to be three in previous studies (Ferguson and Strachan 1982; Wu and Huang 1995; Schrire and Sims 1997). A 4-colporate pollen grain of *I. demissa* Taub. was reported by Ferguson and

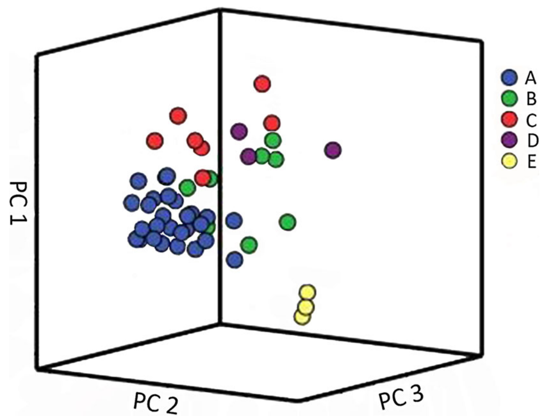

Fig. 4 Scatter plot of the first three components from principal component analysis (PCA); members of the different groups generated from the CA were marked as *different colors*

Strachan (1982), and 4-colporate pollen grains were reported in Taiwan *Indigofera* species (Wu and Huang 1995), but no illustrations or descriptions were available. In this study, pollen grains with four colpi were examined in eight species. Additionally, parasyncolpate pollen grains were examined in four species for the first time. *Indigofera chaetodonta* has the pollen grains with the number of apertures ranging from three to twelve (Fig. 1d).

Variations in the number and arrangement of apertures have been reported as the most common type of variation within the eudicots (Ressayre et al. 2002; Fukuda et al. 2008; Golshan et al. 2014), which can occur at the population level (polymorphism) or at the individual level (heteromorphism) (Walker and Doyle 1975). In this study, aperture variations were observed to be restricted to the individual level. Aperture variations can be caused by genetic factors and environmental conditions, such as the ploidy level, temperature, humidity, latitude, altitude, and pollinator activity (Thanikaimoni 1986; Bredenkamp and Van Wyk 1996; Till-Bottraud et al. 1995; do Pico and Dematteis 2010). For aperture variations in *Indigofera*, we speculated that polyploidization might be one possible reason, especially in *I. decora* complex. In this study, four species of *I. decora* complex (*I. carlesii, I. decora*, *I. decora* var. *ichangensis* and *I. jikongensis*) exhibit some variations in the number of apertures. *Indigofera decora* was reported to be hexaploid which is rare in *Indigofera* (Fedorov 1969), and polyploidization played an important role in the speciation of *I. decora* complex (Choi and Kim 1997). In this study, parasyncolpate pollen grains with 4–12 apertures were observed in one sample of *I. chaetodonta*. *Indigofera chaetodonta* is a subshrub species distributed in Yunnan Province, and the sample was collected from a dried limestone slope in Yulong County, Yunnan Province. Variations in the number of aperture observed in the sample might be triggered by the environmental factors, such as drought, poor and rocky soil in the region.

Exine

Congruent with all previous palynological studies of *Indigofera* (Ferguson and Strachan 1982; Wu and Huang 1995; Schrire and Sims 1997), we found that exine characteristics are highly stable at the infraspecific level. Combining with the phylogeny of Indigofereae (Schrire et al. 2009), we found that pollen grains of species in the Palaeotropical clade (*I. wightii* Graham ex Wight & Arn.; Fig. 2k and *I. colutea*; Fig. 1e) and Tethyan clade (*I. linnaei*; IV: f) have the finely microperforate tectum. Pollen grains of species in Pantropical clade (Sino-Himalayan region) show high diversity level of tectum architecture, which is consistent with the high level of morphological diversity of *Indigofera* in the Sino-Himalayan region. Perforation density and perforation type (especially pitted perforate and pitted microperforate) provide useful information in distinguishing species in Sino-Himalayan region (Table 1). Exine is a stable character in *Indigofera*, and it also showed some phylogenetic and biogeographical significance in *Indigofera*. Exine characteristics including tectum architecture, perforation type and density can be treated as valuable taxonomic characters in *Indigofera*.

Taxonomic significance of pollen characters in *Indigofera*

Indigofera is a large and complex genus taxonomically. Sino-Himalayan region has the highest diversity level of *Indigofera* species in China. The infrageneric classification of Chinese *Indigofera* (Fang and Zheng 1989, 1994) is controversial. The pollen morphology is partly consistent with the infrageneric classification of Fang and Zheng (1989, 1994). For example, species of subsect. *Decorae* (*I. carlesii*, *I. decora*, *I. decora* var. *ichangensis*, *I. jikongensis* and *I. parkesii* Craib) share the similar microperforate tectum in the apocolpium area and pitted microperforate tectum in the mesocolpium. Additionally, pollen grains with more than three apertures were observed in *I. carlesii* (II: d), *I. decora* var. *ichangensis* (III: a) and *I. jikongensis* (IV: d). However, most of the subsections are not supported by pollen characters. Several sister species did not show similar pollen morphology [e.g., *I. szechuensis* Craib (VII: a) and *I. calcicola* Craib (II: c)], while several species that do not have close relationships show similar pollen morphology [e.g., *I. hirsuta* (Fig. 2h) and *I. pseudoheterantha* X.F.Gao & Schrire (V: f)]. Those results indicated that macromorphology and pollen morphology might evolve at different diversification patterns and rates in *Indigofera*. Palynological data also provide further significant evidence for the newly published species (*I. pseudonigrescens*; Fig. 2i) (Zhao and Gao 2015). Pollen morphology of *I. pseudonigrescens* differs from both its morphologically similar species (*I. nigrescens* Kurz ex King & Prain) (Wu and Huang 1995) and phylogenetically related species (*I. delavayi* Franch.; Fig. 1f) in pollen size, the number of apertures, tectum architecture and perforation density.

Given the above results, it is clear that pollen characters alone are insufficient to reconstruct taxonomic relationships within *Indigofera*, but the variations of pollen are useful for the further taxonomic revisions at the species level.

Conclusions

Our study focused on pollen morphology of Chinese *Indigofera*, especially on Sino-Himalayan endemisms. Pollen morphology shows relatively high homogeneity in the examined Chinese *Indigofera* species. The number of apertures varied from 3-colporate type, to 4-colporate type, and the apomorphic (parasyncolpate) types that were observed for the first time. The number of apertures, tectum architecture and perforation density can provide palynological evidence in the taxonomy of *Indigofera*, and these characters also exhibit relatively obvious phylogenetic and biogeographical significance on large scales in *Indigofera*. But pollen shape and size show strong variations and, therefore, are not suitable to be used for taxonomic purposes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Appendix

Voucher information of *Indigofera* species included in this study.

Indigofera amblyantha Craib 1, China, Sichuan: Maoxian, *X. F. Gao* and *X. L. Zhao 15878-9* (CDBI). ***I. amblyantha*** 2, China, Shannxi: Shiquan, *X. L. Zhao* and *C. Q. Peng 208-9* (CDBI). ***I. amblyantha*** 3, China, Zhejiang: Linan, *X. L. Zhao* and *C. Q. Peng 95-13* (CDBI). ***I. argutidens*** Craib, China, Yunnan: Yulong, *X. F. Gao* et al. *9618* (CDBI). ***I. atropurpurea*** Buch.-Ham. ex Hornem., China, Guizhou: Wangmo, *Z. M. Zhu* and *W. B. Ju 397-5* (CDBI). ***I. balfouriana*** Craib, China, Yunnan: Yulong, *X. L. Zhao* et al. *2014-64-4* (CDBI). ***I. bracteata*** Graham ex Baker, China, Xizang: Jilong, *X. F. Gao* et al. *14709* (CDBI). ***I. bungeana*** Walp. 1, China, Sichuan: Maoxian, *X. F. Gao* and *X. L. Zhao 15868-1* (CDBI). ***I. bungeana*** 2, China, Guizhou: Dafang, *Z. M. Zhu* and *W. B. Ju 312-12* (CDBI). ***I. bungeana*** 3, China, Zhejiang: Linan, *X. L. Zhao* and *C. Q. Peng 94-1* (CDBI). ***I. bungeana*** 4, China, Jiangsu: Jurong, *X. L. Zhao* and *C. Q. Peng 63-4* (CDBI). ***I. calcicola*** Craib, China, Sichuan: Derong, *X. F. Gao* et al. *9577-26* (CDBI). ***I. carlesii*** Craib, China, Hubei: Xiangyang, *X. L. Zhao* and *C. Q. Peng 33* (CDBI). ***I. cassioides*** Rottler ex DC., China, Yunnan: Yuanjiang, *X. F. Gao* and *B. Xu 10077* (CDBI). ***I. chaetodonta*** Franch. 1, China, Yunnan: Yulong, *X. F. Gao* et al. *9616-4* (CDBI). ***I. chaetodonta*** 2, China, Yunnan: Yulong, *X. L. Zhao* et al. *2014-43-8* (CDBI). ***I. colutea*** (Burm.f.) Merr., China, Hainan: Sanya, *X. L. Zhao 215-4* (CDBI). ***I. decora*** Lindl., China, Anhui: Jinzhai, *X. L. Zhao* and *C. Q. Peng 56* (CDBI). ***I. decora*** var. ***ichangensis*** (Craib) Y.Y.Fang & C.Z.Zheng, China, Henan: Xinyang, *X. L. Zhao* and *C. Q. Peng 35* (CDBI). ***I. delavayi*** Franch., China, Sichuan: Muli, *X. F. Gao* et al. *9689-19* (CDBI). ***I. dolichochaete*** Craib, China, Yunnan: Eryuan, *X. L. Zhao* et al. *2014-27* (CDBI). ***I. dumetorum*** Craib, China, Yunnan: Yongsheng, *X. L. Zhao* et al. *2014-20* (CDBI). ***I. esquirolii*** H. Lév., China, Guizhou: Wangmo, *Z. M. Zhu* and *W. B. Ju 396-2* (CDBI). ***I. franchetii*** X.F.Gao & Schrire, China, Sichuan: Shimian, *X. F. Gao* et al. *11616-5* (CDBI). ***I. hancockii*** Craib, China, Sichuan: Dechang, *X. L. Zhao* et al. *2014-10-6* (CDBI). ***I. hebepetala*** var. ***glabra*** Ali, China, Xizang: Jilong, *X. F. Gao* et al. *14753* (CDBI). ***I. hendecaphylla*** Jacq., China, Yunnan: Baoshan, *X. F. Gao* et al. *11494* (CDBI). ***I. henryi*** Craib, China, Yunnan: Yulong, *X. F. Gao* et al. *9628-10* (CDBI). ***I. heterantha*** Wall., China, Yunnan: Xianggelila, *X. F. Gao* et al. *9579-2* (CDBI). ***I. hirsuta*** L., China, Hainan: Sanya, *X. L. Zhao 220-1* (CDBI). ***I. jikongensis*** Y.Y.Fang & C.Z.Zheng, China, Henan: Xinyang, *X. L. Zhao* and *C. Q. Peng* 53 (CDBI). ***I. lenticellata*** Craib, China, Sichuan: Luding, *X. F. Gao* et al. *9738-12* (CDBI). ***I. linnaei*** Ali, China, Hainan: Sanya, *X. L. Zhao 216-8* (CDBI). ***I. mairei*** Pamp., China, Sichuan, Luding, *X. F. Gao* et al. *9367-22* (CDBI). ***I. megaphylla*** X.F.Gao, China, Yunnan: Yuanjiang, *X. F. Gao* and *B. Xu 10057-1* (CDBI). ***I. pampaniniana*** Craib, China, Yunnan: Shiping, *X. F. Gao* et al. *10086-29* (CDBI). ***I. parkesii*** Craib, China, Zhejiang: Tiantai, *X. L. Zhao* and *C. Q. Peng 81* (CDBI). ***I. pendula*** Franch., China, Yunnan: Yongsheng, *X. L. Zhao* et al. *2014-14* (CDBI). ***I. penduloides*** Y.Y.Fang & C.Z.Zheng, China, Yunnan: Yulong, *B. Xu* and *A. N. Egan 459* (CDBI). ***I. pseudoheterantha*** X.F.Gao & Schrire, China, Sichuan: Miyi, *X. F. Gao* et al. *9748* (CDBI). ***I. pseudonigrescens*** X.F.Gao & Xue Li Zhao, China, Sichuan: Mianning, *X. L. Zhao* et al. *2014-1-1* (CDBI). ***I. rigioclada*** Craib, China, Yunnan: Yulong, *X. F. Gao* et al. *9610-26* (CDBI). ***I. scabrida*** Dunn, China, Yunnan: Yulong, *X. F. Gao* et al. *9609-6* (CDBI). ***I. sensitiva*** Franch., China, Sichuan: Muli, *X. L. Zhao* et al. *2014-123-2* (CDBI). ***I. silvestrii*** Pamp. 1, China, Sichuan: Maoxian, *X. F. Gao* and *X. L. Zhao 15893-9* (CDBI). ***I. silvestrii*** Pamp. 2, China, Yunnan: Weixi, *X. L. Zhao* et al. *2014-71-2* (CDBI). ***I. stachyodes*** Lindl., China, Yunnan: Yuanjiang, *X. F. Gao* and *B. Xu 10079-11* (CDBI). ***I. sticta*** Craib, China, Yunnan: Yuanjiang, *X. F. Gao* and *B. Xu 10073-25* (CDBI). ***I. suffruticosa*** Mill., China, Hainan: Ledong, *X. L. Zhao 235-2* (CDBI). ***I. szechuensis*** Craib, China, Sichuan: Maoxian, *X. F. Gao* and *X. L. Zhao 15912-1* (CDBI). ***I. wightii*** Graham ex Wight & Arn., China, Hainan: Ledong, *X. L. Zhao 236-1* (CDBI). ***I. wilsonii*** Craib, China, Sichuan: Wenchuan, *X. F. Gao* and *X. M. Wei 6-1* (CDBI).

Information on Electronic Supplementary Material

Online Resource 1. SEM micrographs of pollen grains in *Indigofera*.
Online Resource 2. Comparisons of pollen shape of pollen grains from the flowers with different maturity.

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