



Palynological characters and their systematic significance in Naucleaeae (Cinchonoideae, Rubiaceae)

YanFeng Kuang^{a,b,d}, Bruce K. Kirchoff^c, Yuanjiang Tang^{a,b}, YuanHui Liang^a, JingPing Liao^{a,b,*}

^a South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, 510650, China

^b Key Laboratory of Digital Botanical Garden in Guangdong, Guangzhou, 510650, China

^c Department of Biology, University of North Carolina at Greensboro, 312 Eberhart, P. O. Box 26170, Greensboro, NC 27412, USA

^d Graduate School of Chinese Academy of Sciences, Beijing, 100039, China

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ABSTRACT

Phylogenetic studies have improved Naucleaeae classification, but the relationships among the subtribes remain largely unresolved. This can be explained by the inadequate number of synapomorphies shared among these lineages. Of the 49 morphological characters used in phylogenetic analyses, none were from pollen. It has been proposed that H-shaped endoapertures form a synapomorphy of the Naucleaeae. Further study of Naucleaeae pollen is needed to test this hypothesis as the endoapertures of many Naucleaeae genera are unknown.

Pollen morphology of 24 species was examined using scanning electron and light microscopy. Naucleaeae pollen is very small to small, with a spheroidal to subprolate shape in equatorial view. Three compound apertures are present, each comprised of a long ectocolpus, a longolate to (sub)circular mesoporus, and an often H-shaped endoaperture. The sexine ornamentation is microreticulate to striate, rugulate, or perforate. Pollen wall ultrastructure of five species was studied with transmission electron microscopy. The exine is composed of a perforated tectum, short columellae, and a thick nexine. The nexine is often differentiated into a foot layer and an endexine, and thickened into costae towards the aperture. The intine often protrudes from the aperture forming a protruding oncus. Our observations support the phylogenetic delimitation of the Naucleaeae *sensu* Razafimandimbison and Bremer, but pollen morphology is of little value in distinguishing the subtribes and genera of the Naucleaeae.

Ancestral state reconstruction using MacClade is unambiguous in showing that the possession of an H-shaped endoaperture and protruding onci (a new character for the tribe) form morphological synapomorphies of the clade *Hymenodictyon*+ Naucleaeae.

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1. Introduction

The Naucleaeae are a predominantly palaeotropical tribe of the subfamily Cinchonoideae (Rubiaceae) comprising 26 genera and 179 species (Razafimandimbison and Bremer, 2002). The center of distribution of the tribe is Southeast Asia (Ridsdale, 1978). Members of the Naucleaeae are characterized by numerous-flowered globose inflorescences, and epigynous floral nectaries deeply embedded in hypanthia. Members of the tribe occur in various habitats ranging from terrestrial (rainforests, deciduous dry forests, and savannas) to wet (swampy forests and stable or running rivers) (Bremer et al., 1995; Razafimandimbison and Bremer, 2001, 2002; Razafimandimbison, 2002).

The intratribal classification of the Naucleaeae is controversial (Haviland, 1897; Verdcourt, 1958; Bremekamp, 1966; Ridsdale, 1978;

Robbrecht, 1994). In order to test the monophyly of previous subtribal circumscriptions, Razafimandimbison and Bremer (2001, 2002) conducted phylogenetic analyses based on three molecular data sets (ITS, rbcL, trnT-F), and 49 morphological characters. Their results strongly suggest a much broader circumscription for the Naucleaeae than previously proposed, including not only all members of the Naucleaeae *sensu* Ridsdale, but also *Cephalanthus*, *Hallea*, *Mitragyna*, *Uncaria*, *Corynanthe* and *Pausinystalia*. Their analyses also showed that the Naucleaeae can be subdivided into six highly supported and morphologically distinct subtribes: Breoniinae, Cephalanthinae, Corynantheinae, Naucleinae, Mitragyninae, and Uncarinae. A seventh tribe Adininae, is poorly supported. The Cephalanthinae occur in a basal position, and are sister to the remaining subtribes, which are placed in a large clade. Unfortunately, the relationships among the subtribes of this clade are largely unresolved. This can be explained by the inadequate number of synapomorphies shared among these lineages, which are mostly united by homoplastic characters. Of the 49 morphological characters used in the analyses, no pollen morphological characters are included.

* Corresponding author. South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, 510650, China. Tel./fax: +86 20 8523 1520.

E-mail address: liaojp@scbg.ac.cn (J. Liao).

Pollen morphological characters have proved to be particularly informative in elucidating evolutionary relationships in many groups of Rubiaceae (e.g., Johansson, 1987; Andersson, 1993; Persson, 1993; Rova and Andersson, 1995; Delprete, 1996; De Block and Robbrecht, 1998; Huysman et al., 1998; Huysmans et al., 1999; Dessein et al., 2002). They are frequently incorporated into morphological cladistic analyses, and can be useful in supporting or rejecting molecular phylogenetic hypotheses (Huysmans et al., 1994; Piesschaert et al., 2000; Dessein et al., 2005a).

Pollen morphology of the Naucleaeae, was first investigated by Leroy (1975), who conducted an extensive study of *Hallea* and *Mitragyna* pollen. Huysmans et al. (1994) completed this work by investigating the pollen of all ten species of these genera. They concluded that the genera could not be recognized based solely on pollen morphology. Pollen morphology of ten species belonging to seven genera occurring in China was briefly described by Liang (1982) and Wang et al. (1995). More recently, Verellen et al. (2007) have surveyed Naucleaeae pollen. Their results support the broader delimitation of the Naucleaeae *sensu* Razafim. and Bremer, but cannot provide unambiguous support for subtribal or generic delimitations because of lack of variation in pollen characters. Verellen et al. (2007) also proposed that an H-shaped endoaperture forms a synapomorphy of the Naucleaeae. Further study of the Naucleaeae pollen is needed to test this hypothesis, as the endoaperture of many Naucleaeae genera remains unknown. The goals of the present paper are: (1) to complement existing palynological data on the Naucleaeae including surveying for the presence of a protruding oncus, a character that has been reported in other Rubiaceae taxa; (2) to test the hypothesis that the possession of H-shaped endoapertures forms a synapomorphy of the tribe; (3) to find additional pollen morphological synapomorphies for the tribe.

2. Materials and methods

Forty-two collections of 22 species from 11 genera of the Naucleaeae, and of two species of *Hymenodictyon* occurring in China were examined (Table 1). Polliniferous anthers were collected from living plants growing in the South China Botanical Garden (SCBG), and/or from herbarium specimens from the following herbaria: IBSC, KUN and PE. Voucher specimens of the fresh material are deposited in IBSC (Table 1).

For light (LM) and scanning electron microscopy (SEM), the polliniferous anthers were softened by soaking in glacial acetic acid (Reitsma, 1969), and dissected with tweezers to release the pollen. The separation of the pollen from the remaining anther material was accomplished with a sieve (pore diameter 50 µm). Each pollen sample was then split into two parts. One part was acetolysed (three to five minutes in a heating block at 98 °C) according to the method of Erdtman (1960) and, transferred to 70% ethanol. The other part was transferred directly to 70% ethanol. Both parts were washed three times in 70% ethanol in an ultrasonic bath, for ten minutes each time. For LM, the pollen was mounted on slides in glycerine jelly, and coverslips sealed to the slides with paraffin. LM photographs were taken at a magnification of 1000×. For SEM, the pollen was mounted on copper stubs, air-dried, and coated with gold in a JFC-1600 sputter coater (JEOL Ltd, Tokyo, Japan). Observations and digital images were collected with a JEOL JSM-6360LV SEM (JEOL Ltd, Tokyo, Japan).

Pollen wall ultrastructure was investigated with fresh pollen from *Adina pilulifera*, *Neolamarckia cadamba*, and *Uncaria hirsuta*, and pollen from herbarium specimens of *Metadina trichotoma*, and *Pertusadina hainanensis*. For transmission electron microscopy (TEM), hydrated, unacetolysed pollen was fixed in 2.5% glutaraldehyde at pH 7.2, rinsed in 0.1 M phosphate buffer for 2 h, then postfixed in 1% osmium tetroxide for 2 h or more. The pollen was then washed in phosphate buffer, dehydrated in an acetone series, embedded in Spurr's resin, and cured at 70 °C. Ultrathin sections (80 nm) were cut using a Leica-Ultracut S ultramicrotome (Leitz Inc., Wiesbaden, Germany), and stained with

Table 1
The collections examined

Species	Collection	Locality	Herbarium
<i>Adina pilulifera</i> (Lam.) Franch. Ex Drake (2)	Kuang 0004*	Guangdong, China	IBSC
	s.n. 34039	Guangdong, China	IBSC
<i>Adina rubella</i> Hance (2)	Kuang 1001*	Guangdong, China	IBSC
	Liu 24842	Guangdong, China	IBSC
<i>Cephalanthus tetrandra</i> (Roxb.) Ridsd. and Bakh. f. (2)	Tam 861	Guangdong, China	IBSC
	Liu 00679	Guangdong, China	IBSC
<i>Haldina cordifolia</i> (Roxb.) Ridsd. (3)	Tao 38599	Yunnan, China	KUN
	Hu 512	Yunnan, China	IBSC
	Xiao 89	Yunnan, China	IBSC
<i>Metadina trichotoma</i> (Zoll. and Moritz) Bakh. f. (2)	SCFU 30992	Hunan, China	IBSC
	s.n. 22309	Guangxi, China	IBSC
<i>Mitragyna rotundifolia</i> (Roxb.) Kuntze (2)	Mao 6879	Yunnan, China	IBSC
	Wang 74334	Yunnan, China	IBSC
<i>Nauclea officinalis</i> (Pierre ex Pit.) Merr. and Chun (3)	Mao 6742	Yunnan, China	IBSC
	s.n. 75305	Philippines	IBSC
	Kuang 2001*	Guangdong, China	IBSC
<i>Neolamarckia cadamba</i> (Roxb.) Bossler (1)	Kuang 0006*	Guangdong, China	IBSC
<i>Neonauclea griffithii</i> (Hook. f.) Merr. (1)	Wang 81146	Yunnan, China	IBSC
<i>Neonauclea truncata</i> (Hayata) Yamamoto (2)	Yang et al. 1584485	Taiwan, China	PE
<i>Neonauclea sessilifolia</i> (Roxb.) Merr. (2)	Kewakmi 1660	Taiwan, China	IBSC
	Wang 78006	Yunnan, China	PE
<i>Pertusadina hainanensis</i> (How) Ridsd. (1)	Wang 79875	Yunnan, China	IBSC
	Zuo 21095	Guangdong, China	IBSC
<i>Sinoadina racemosa</i> (Siebole and Zucc.) Ridsd. (2)	Zhong 808182	Guangxi, China	IBSC
<i>Uncaria hirsuta</i> Havil. (2)	Zhong 889182	Guangxi, China	IBSC
	Kuang 1002*	Guangdong, China	IBSC
	Yue 78–6321	Guangdong, China	IBSC
<i>Uncaria laevigata</i> Wall. Ex G. Don (1)	Drake 19191	Yunnan, China	KUN
<i>Uncaria lancifolia</i> Hutchins. (2)	Lu 2396	Guangxi, China	IBSC
	Cai 62226	Yunnan, China	IBSC
<i>Uncaria macrophylla</i> Wall. (2)	Li 01950	Guangxi, China	IBSC
	Wang 78767	Yunnan, China	IBSC
<i>Uncaria rhynchophylla</i> (Miq.) Miq. ex Havil. (2)	Deng 1247	Guangdong, China	IBSC
	Yang 830397	Jiangxi, China	IBSC
<i>Uncaria rhynchophylloides</i> How (1)	Shi 445	Guangdong, China	IBSC
<i>Uncaria scandens</i> (Smith) Hutchins. (1)	How and Chen 70249	Hainan, China	IBSC
<i>Uncaria sessilifrutosa</i> Roxb. (2)	Tam 2610	Yunnan, China	IBSC
	Qin 7648	Guangxi, China	IBSC
<i>Uncaria sinensis</i> (Oliv.) Havil. (2)	Li 3898	Hubei, China	IBSC
	Sin 51083	Guizhou, China	IBSC
<i>Hymenodictyon flaccidum</i> Wall (1)	Li 602987	Guangxi, China	IBSC
<i>Hymenodictyon orixense</i> (Roxb.) Mabb. (1)	Wang 78020	Yunnan, China	IBSC

Explanations: numbers in parentheses after each species' name is the number of collections investigated of that species. Collections indicated with an asterisk were taken from fresh material, the rest are from herbarium specimens.

uranyl acetate and lead citrate. Transmission electron micrographs were taken with a JEM-1010 (JEOL Ltd, Tokyo, Japan) transmission electron microscope at 100 KV.

Measurements of the polar axis (*P*) and equatorial diameter (*E*) were made with LM from 10–20 pollen grains per specimen. All other measurements were made on digital SEM images with JEOL's Smile View 2.2.6.1 software (JEOL Ltd, Tokyo, Japan). The colpi/polar axis ratio multiplied by 100 ($=LC/P \times 100$) was used to express the relative length of the colpi. Measurements of ectocolpus width refer to the transverse diameter of the mesoporus, if present, or to the widest opening of the ectocolpus, if there is no mesoporus. Measurements of the layers of the pollen wall were made on 10–20 TEM images (10–20 pollen grains) of each taxon.

Palynological terminology follows that of Punt et al. (2007). Pollen size, and shape classes in equatorial view refer to Erdtman (1969). The generic delimitations and infratribal taxa adopted here are as circumscribed by Razafimandimbison and Bremer (2002). Unfortunately, these authors did not place the two genera *Diyaminauclea* and *Khasiaclunea*, and the two genera *Haldina* and *Sinoadina* are only provisionally accommodated in the poorly supported subtribe Adinia.

In order to investigate their potential as synapomorphies, the characters “H-shaped endoaperture” and “protruding oncus” were mapped on the phylogenetic tree of the Naucleaeae-Hymenodictyoneae clade (Razafimandimbison and Bremer, 2001, 2002), and their ancestral states were reconstructed from unordered characters with the software MacClade 4.06 (Maddison and Maddison, 2003). In the analyses of Razafimandimbison and Bremer (2001, 2002) the genus *Luculia*, which had been shown to be basal in the Rubiaceae by Bremer et al. (1999), was used to root the tree while the genera *Exostema* and *Cinchona* were used as additional outgroups. For our analyses we replaced the terminals (species) of Razafimandimbison and Bremer (2001, 2002) with the corresponding genera. The occurrence of H-shaped endoapertures in a genus, whether distinct, indistinct or incomplete, were coded as the character state “H-shaped endoapertures present,” regardless of whether or not they occur in all members of a genus. For example, H-shaped endoapertures are coded as present in *Uncaria*, even though they have only been reported in *U. rhynchophylla*. Endoapertures in other patterns are coded as “H-

shaped endoapertures absent.” Distinct and indistinct protruding onci, and protruding oncus remnants, are coded as the character state “protruding oncus present,” regardless of whether or not they occur in all members of a genus.

3. Results

3.1. General features

The pollen grains are always monads, radiosymmetric, and very small ($E < 10 \mu\text{m}$) to small ($E 10\text{--}25 \mu\text{m}$). Their shape in equatorial view is spheroidal (*P/E* 0.88–1.14) to subprolate (*P/E* 1.14–1.33). The spheroidal condition can be subdivided into oblate spheroidal (*P/E* 0.88–1), and prolate spheroidal (*P/E* 1–1.14). The outline in polar view (amb) is usually (sub)circular with sunken colpi. Three compound apertures are found in each grain, each comprised of a long ectocolpus, a lolongate to slightly circular mesoporus, and an often H-shaped endoaperture. H-shaped endoapertures are the inner of a three-part compound aperture, and have two cross members, one above and one below the porus. The ectocolpus membrane is usually granular, though it is not visible in some species due to the narrowness of the ectocolpus. The mesoporus is always located in the middle of the ectocolpus, at the equator. The sexine ornamentation is microreticulate to striate, rugulate, or perforate; the lumina of microreticulations are usually irregularly polygonal and (sub)circular. There is usually no differentiation of the sexine towards the poles and/or colpi. Exine and intine are both obvious under LM and TEM. In TEM, the exine is composed of a perforated tectum, relatively short columellae, and a thick nexine. The nexine is often differentiated into a foot layer and an endexine, and thickened into costae towards the aperture. The intine of some species is thickened in the apertural region, and protrudes from the aperture forming a protruding oncus (Plate IV). This character can be variable in taxa in which it occurs, so that some grains possess it and others do not.

Table 2

Pollen morphological characters for each species involved in this research

Species	Size			Shape	Ectocolpus		Apocolpium index	Sexine ornamentation
	<i>P</i>	<i>E</i>	<i>P/E</i>		Width	LC/ <i>P</i>		
	(μm)	(μm)			(μm)			
<i>Neonauclea griffithii</i>	10.5 (9.4–11.9)	10.9 (9.7–12.6)	0.96	OS	0.59	73	0.3	Microreticulate
<i>Neonauclea sessilifolia</i>	11.3 (9.9–11.6)	12.0 (10.9–13.6)	0.94	OS	1.22	62	0.53	Microreticulate
<i>Neonauclea truncata</i>	11.7 (9.8–12.2)	12.4 (11.7–13.3)	0.94	OS	1.15	71	0.43	Microreticulate
<i>Uncaria hirsuta</i>	9.4 (8.8–10.4)	10.7 (9.7–11.9)	0.88	OS	0.32	73	0.37	Striate-reticulate
<i>Uncaria laevigata</i>	12.4 (11.5–13.5)	12.7 (11.2–13.5)	0.98	OS	1.87	87	0.25	Striate-reticulate
<i>Uncaria lancifolia</i>	11.5 (10.3–12.7)	11.0 (10.4–11.9)	1.04	PS	0.58	65	0.38	Striate-reticulate
<i>Uncaria macrophylla</i>	10.3 (8.5–11.8)	9.0 (8.6–9.3)	1.14	PS	0.33	77	0.33	Striate-reticulate
<i>Uncaria rhynchophylla</i>	12.0 (11.3–13.2)	9.9 (9.0–11.2)	1.21	SP	0.45–1.25	77	0.42	Striate-reticulate
<i>Uncaria rhynchophylloides</i>	12.2 (11.2–13.1)	11.1 (10.3–12.3)	1.1	PS	0.52	73	0.43	Striate-reticulate
<i>Uncaria scandens</i>	11.7 (10.5–12.7)	10.1 (9.0–11.5)	1.16	SP	0.76	71	0.35	Striate-reticulate
<i>Uncaria sessilifrutus</i>	10.2 (9.5–10.8)	9.8 (9.3–10.7)	1.03	PS	0.28	61	0.39	Striate-reticulate
<i>Uncaria sinensis</i>	12.6 (11.1–14.2)	10.9 (9.4–12.1)	1.15	SP	0.73–1.41	76	0.39	Striate-reticulate
<i>Adina pilulifera</i>	9.7 (9.2–10.6)	10.7 (9.9–11.9)	0.93	OS	0.85	73	0.37	Rugulate-microreticulate
<i>Adina rubella</i>	11.0 (9.9–12.1)	11.0 (9.5–11.7)	1.01	PS	1.09	74	0.39	Rugulate-microreticulate
<i>Cephalanthus tetrandra</i>	14.7 (13.3–15.8)	15.9 (14.8–17.3)	0.93	OS	1.98	80	0.26	Perforate
<i>Haldina cordifolia</i>	12.5 (11.6–14.3)	11.9 (11.2–13.4)	1.05	PS	2.29	82	0.3	Microreticulate
<i>Metadina trichotoma</i>	12.1 (10.7–13.3)	9.93 (8.8–11.0)	1.22	SP	0.99	79	0.32	Perforate-microreticulate
<i>Mitragyna rotundifolia</i>	12.1 (10.7–13.6)	12.0 (10.4–13.1)	1.01	PS	2.12	73	0.36	Microreticulate
<i>Nauclea officinalis</i>	11.8 (11.4–12.1)	11.1 (9.6–13.4)	1.06	PS	1.95	66	0.44	Rugulate-microreticulate
<i>Neolamarckia cadamba</i>	9.7 (9.2–10.6)	10.7 (9.9–11.9)	0.93	OS	1.24	75	0.36	Striate-reticulate
<i>Pertusadina hainanensis</i>	12.3 (10.9–14.9)	10.0 (8.8–11.2)	1.24	SP	0.96	78	0.36	Microreticulate
<i>Sinoadina racemosa</i>	12.0 (10.7–13.5)	10.4 (9.2–11.8)	1.15	SP	0.5–1.24	75	0.3	Rugulate-striate
<i>Hymenodictyon flaccidum</i>	12.4 (11.7–12.8)	10.2 (9.5–10.7)	1.22	SP	1.21	79	0.31	Microreticulate-perforate
<i>Hymenodictyon orixense</i>	12.2 (11.5–12.9)	10.7 (9.8–11.1)	1.21	SP	0.72	75	0.29	Microreticulate-perforate

Explanations: *E*, equatorial diameter; *P*, polar axis diameter; *LC/P*, the length colpi/polar axis ratio multiplied by 100; Apocolpium index, the ratio of the distance between the apices of two ectocolpus of a zonocolpate pollen grain to its equatorial diameter; OS, oblate spheroidal; PS, prolate spheroidal; SP, subprolate.

3.2. Detailed descriptions (Table 2)

Generic descriptions are given for two genera, (*Neonauclea*, and *Uncaria*) in order to concisely indicate the variation among the species investigated in these genera. Generic descriptions of the other ten genera are not given because only one or two species were studied in each genus. The pollen morphology of nine species (indicated with asterisks, e.g., *Neonauclea sessilifolia**) belonging to four genera is reported for the first time. The remaining 15 species (e.g., *Neonauclea griffithii*, without any symbol) belonging to 11 genera are described only by important palynological characters that have not been reported previously (Table 2).

Neonauclea Merr. (65 species; 3 investigated): Pollen very small to small, *P* 10.5 (9.4–1.9) μm , *E* 10.9 (9.7–12.6) μm ; shape in equatorial view oblate spheroidal, amb slightly triangular, subcircular to circular. Apertures 3; colporate, with long, narrow ectocolpi, each 0.59–1.22 μm wide, and with a relative length ranging from 62 (*Neonauclea sessilifolia*) to 73 (*Neonauclea griffithii*), ends acute, membrane granular; mesoporus subcircular to lologate; incompletely H-shaped endoaperture presents in *N. sessilifolia*; protruding onci occur in *N. griffithii*. Sexine sculpture microreticulate, without differentiation towards the poles and colpi, lumina of microreticulations irregularly oblong or polygonal. There is little variation in pollen size, shape, aperture number, or sexine ornamentation in the genus.

N. griffithii (Plate I, A; Plate III, A): Ectocolpus ends acute, membrane granular; mesoporus subcircular, with protruding oncus; endoaperture not H-shaped; lumina of microreticulations irregularly oblong or polygonal.

*N. sessilifolia** (Plate I, B–C; Plate III, B): Ectocolpus ends acute to obtuse, membrane granular; mesoporus lologate (Plate III, B), protruding oncus absent; endoaperture faint, incompletely H-shaped (Plate III, B). Sexine sculpture rugulate to microreticulate (Plate I, C), without differentiation towards the colpi, but with slightly larger lumina at poles, lumina irregularly oblong or polygonal.

*N. truncata** (Plate I, D; Plate III, C): Ectocolpus ends acute to obtuse, membrane granular; mesoporus lologate (Plate III, C), protruding oncus absent; endoaperture not H-shaped; lumina of microreticulations irregularly oblong or polygonal.

Uncaria Schreber (34 species; 9 investigated) Pollen very small to small, *P* (8.5–13.8) μm , *E* (8.6–13.5) μm ; shape in equatorial view spheroidal to subprolate, the spheroidal condition subdivided into oblate spheroidal (*P/E* 0.88–1) and prolate spheroidal (*P/E* 1–1.14), amb circular with three lobes due to the sunken colpi. Apertures 3; colporate, with long and very narrow (slit-like) ectocolpi, width ranging from 0.28 μm (*Uncaria sessilifrustrus*) to 1.87 μm (*Uncaria laevigata*), relative length 61–87, ends acute to obtuse, membrane granular, although not visible in some species; mesoporus circular to lologate; indistinctly H-shaped endoaperture present in *U. rhynchophylla*, endoapertures in other species not H-shaped; in some species distinct or indistinct protruding onci present. The sexine pattern may equally well be described as striate to reticulate with interwoven muri, or rugulate with slender, long striae on the reticulum, without differentiation towards poles or colpi; lumina of microreticulations irregularly oblong or subcircular. There is little variation in pollen size, shape, sexine ornamentation and aperture morphology in this genus.

U. hirsuta (Plate I, E–F; Plate III, D): Ectocolpus ends acute, membrane not visible; mesoporus lologate (Plate III, D), distinct protruding oncus present, (Plate I, E–F); endoaperture not H-shaped; lumina of microreticulations irregularly oblong or subcircular.

*U. laevigata** (Plate I, G): Ectocolpus ends acute to obtuse, membrane granular; mesoporus circular to lologate, protruding oncus present; endoaperture unknown; lumina of microreticulations irregularly oblong or subcircular.

*U. lancifolia** (Plate I, H; Plate III, E): Ectocolpus ends acute, membrane granular; mesoporus lologate (Plate III, E), protruding

oncus absent; endoaperture not H-shaped; lumina of microreticulations irregularly oblong or subcircular.

U. macrophylla (Plate I, I; Plate III, F): Ectocolpus ends acute, membrane not visible; mesoporus subcircular, with indistinct protruding oncus (Plate III, F); endoaperture not H-shaped; lumina of microreticulations irregularly oblong or subcircular.

U. rhynchophylla (Plate I, J; Plate III, G): Ectocolpus ends acute to obtuse, membrane granular; mesoporus subcircular (Plate III, G), protruding oncus present (Plate I, J); endoaperture faint, indistinctly H-shaped (Plate III, G); lumina of microreticulations irregularly oblong or subcircular.

*U. rhynchophylloides** (Plate I, K): Ectocolpus ends acute, membrane not visible; mesoporus subcircular, without protruding oncus; endoaperture unknown; lumina of microreticulations irregularly oblong or subcircular.

*U. scandens** (Plate I, L; Plate III, H): Ectocolpus ends acute, membrane granular; mesoporus subcircular (Plate III, H), protruding oncus present (Plate I, L); endoaperture not H-shaped; lumina of microreticulations irregularly oblong or subcircular.

U. sessilifrustrus (Plate I, M–N; Plates III, I): Ectocolpus ends acute, membrane not visible; mesoporus lologate, with protruding oncus (Plates III, I); endoaperture not H-shaped; lumina of microreticulations irregularly oblong or subcircular.

*U. sinensis** (Plate I, O; Plate III, J): Ectocolpus ends acute, membrane granular; mesoporus subcircular (Plate III, J), protruding oncus present (Plate I, O; Plate III, J); endoaperture not H-shaped; lumina of microreticulations irregularly oblong or subcircular.

Adina pilulifera (Plate II, A; Plate III, K): Ectocolpus ends acute, membrane granular; mesoporus circular (Plate III, K), with protruding oncus or oncus remnants (Plate II, A); endoaperture faint, indistinctly H-shaped; lumina of microreticulations irregularly polygonal.

A. rubella (Plate II, B): Ectocolpus ends acute, membrane granular; mesoporus subcircular, protruding oncus present; endoaperture unknown; lumina of microreticulations irregularly polygonal.

Cephalanthus tetrandra (Plate II, C–D; Plate III, L): Ectocolpus protuberant in the middle (Plate II, C), ends acute, membrane granular; mesoporus circular (Plate III, L), with indistinct protruding oncus (Plate II, C); endoaperture distinct, clearly H-shaped (Plate III, L); lumina/perforations of microreticulations irregularly subcircular or polygonal.

Haldina cordifolia (Plate II, E–F; Plate III, M): Ectocolpus ends obtuse to acute, membrane granular; mesoporus lologate (Plate III, M), with distinct protruding oncus (Plate II, E); endoaperture faint, incompletely H-shaped; lumina of microreticulations irregularly polygonal.

Metadina trichotoma (Plate II, G; Plate III, N): Ectocolpus ends acute, membrane not visible; mesoporus lologate (Plate III, N), with protruding oncus (Plate II, G); endoaperture distinct, clearly H-shaped (Plate III, N); lumina of microreticulations usually irregularly polygonal.

Mitragyna rotundifolia (Plate II, H; Plate III, O): Ectocolpus ends acute, membrane granular; mesoporus circular (Plate III, O), protruding oncus absent; endoaperture distinct, clearly H-shaped (Plate III, O); lumina/perforations of microreticulations irregularly oblong or polygonal.

Nauclea officinalis (Plates II, I; Plate III, P): Ectocolpus ends acute to obtuse, membrane granular; mesoporus lologate (Plate III, P), with distinct protruding oncus (Plates II, I); endoaperture indistinct, not H-shaped; lumina of microreticulations irregularly polygonal.

Neolamarckia cadamba (Plate II, J): Ectocolpus ends acute, membrane granular; mesoporus circular, distinct protruding oncus present; endoaperture unknown; lumina of microreticulations irregularly polygonal.

*Pertusadina hainanensis** (Plate II, K; Plate III, Q): Ectocolpus protuberant in the middle (Plate II, K), ends acute, membrane not visible; mesoporus circular, with protruding oncus or protruding oncus remnants (Plate III, Q); endoaperture indistinct, not H-shaped; lumina of microreticulations irregularly oblong or polygonal.

Sinoadina racemosa (Plate II, L; Plate III, R): Ectocolpus ends acute, membrane not visible; mesoporus subcircular (Plate III, R), with protruding oncus or remnants (Plate II, L); endoaperture faint, indistinctly H-shaped; lumina of microreticulations irregularly polygonal.

*Hymenodictyon flaccidum** (Plate II, M–N; Plate III, S): Ectocolpus ends acute, membrane granular; mesoporus lolongate, with protruding oncus or remnants (Plate II, N); endoaperture H-shaped (Plate III, S); lumina of microreticulations irregularly oblong or polygonal.

H. orixense (Plate II, O): Ectocolpus ends acute, membrane granular; mesoporus subcircular, protruding oncus absent; endoaperture unknown; lumina of microreticulations irregularly oblong or polygonal.

3.3. Pollen wall ultrastructure

The pollen wall of the five species investigated by TEM is composed of a discontinuous tectum, relatively short columellae, a thick nexine, and a thin intine (Plate IV). Exine and intine are both obvious. In all five species the intine becomes thicker in the apertural region, and protrudes from the aperture forming a bubble-like structure, the protruding oncus.

Adina pilulifera (Plate IV, A–B): Tectum 0.2–0.39 μm thick; columellae 0.11–0.25 μm thick; nexine differentiated into a foot layer and endexine, the foot layer separated from the endexine by a single line, which is little different from these layers in electron density. Nexine 0.19–0.28 μm thick in mesocolpial region, becoming thinner near the aperture and then thickened into costae surrounding the aperture. Intine usually 0.07–0.28 μm thick, thicker (ca. 0.69 μm) near the aperture, and forming a protruding oncus.

Metadina trichotoma (Plate IV, C–D): Tectum 0.16–0.35 μm thick; columellae 0.04–0.25 μm thick, electron-dense material (possibly lipidic) occurring occasionally between the columellae. Nexine not differentiated into a foot layer and endexine, usually 0.06–0.24 (–0.61) μm thick in mesocolpial region, thickened into costae around the aperture. Intine usually 0.07–0.38 μm thick, forming protruding onci. A less fibrillar zone occurs beneath the aperturate intine.

Neolamarckia cadamba (Plate IV, E–F): Tectum indistinct, 0.18–0.38 μm thick; columellae indistinct, 0.01–0.05 μm thick, electron-dense material (possibly lipidic and much wider than the columellae), occurring occasionally between columellae. Nexine differentiated into a foot layer and a very thin endexine, the foot layer separated from the endexine by a single white line. Nexine 0.11–0.18 μm thick in mesocolpial region, thickened into costae around the aperture. Intine usually 0.02–0.06 μm thick, forming a protruding oncus ca. 1.6 μm in diameter. Oncus composed of a bi-layered ectintine with an electron-dense outer layer and a thick electron-lucent inner layer; and separated from the cytoplasm of the pollen cell by intine material.

Pertusadina hainanensis (Plate IV, G–H): Tectum 0.16–0.29 μm thick; columellae indistinct, 0.04–0.16 μm thick, electron-dense material (possibly lipidic) occurring occasionally between the columellae. Nexine not clearly differentiated into a foot layer and endexine, 0.07–0.27 μm thick in mesocolpial region, becoming thinner near the aperture, and then thickened into costae surrounding the aperture. Intine usually 0.01–0.18 μm thick in the apertural region, and forming a protruding oncus; oncus composed of a bi-layered ectintine: an electron-dense outer layer, and a thick electron-lucent inner layer.

Uncaria hirsuta (Plate IV, I–J): Tectum 0.16–0.48 μm thick; columellae 0.1–0.16 μm thick, nexine differentiated into a foot layer and endexine, the foot layer separated from the endexine by a single white line. Nexine 0.16–0.35 μm thick in mesocolpial region, becoming thinner near the aperture, and then thickened into costae immediately surrounding the aperture. Intine usually 0.18–0.32 μm thick, forming a subcircular oncus, ca. 2.5 μm in diameter. The oncus is composed of a bi-layered ectintine: an electron-dense outer layer, and a thick electron-lucent inner layer. Cytoplasmic components, e.g., a vacuole (Plate IV, J) and starch (unpublished images), are sometimes found in the onci.

3.4. Ancestral character state reconstruction

3.4.1. H-shaped endoaperture (Fig. 1)

H-shaped apertures occur in all the genera of the subtribes Cephalanthinae, Mitragyninae, Uncarinae, and Corynantheinae, and in five genera (including *Haldina* and *Sinoadina*) of subtribe Adininae. One genus of subtribe Breoniinae also possesses H-shaped endoapertures, while the condition in the other three genera remains unknown. The endoapertures in two genera of the subtribe Naucleinae are not H-shaped; the condition in the other two genera remains unknown. *Hymenodictyon* of the tribe Hymenodictyaceae, the sister group of the Naucleaceae, has H-shaped endoapertures (Fig. 1).

Parsimony reconstruction suggests that all the ancestral nodes in the outgroups lack H-shaped endoapertures, while the ancestor of the clade containing *Hymenodictyon* and the Naucleaceae possesses them. Many of the internal nodes of the Naucleaceae also possess H-shaped endoapertures, though the polytomies make the reconstruction of the more derived nodes difficult.

3.4.2. Protruding oncus (Fig. 2)

Of the 12 genera that have been investigated, protruding onci occur in six genera (including *Haldina* and *Sinoadina*) of subtribe Adininae, two of subtribe Naucleinae, the single genus *Uncaria* of subtribe Uncarinae, the single genus *Cephalanthus* of subtribe Cephalanthinae, and *Hymenodictyon* of tribe Hymenodictyaceae. Only the genus *Mitragyna* s.s. of Mitragyninae lacks this characteristic. The condition in the remaining 13 genera distributed in subtribes Adininae, Breoniinae, Corynantheinae, Mitragyninae, and Naucleinae remain unknown (Fig. 2).

Parsimony reconstruction suggests that a protruding oncus is a synapomorphy of the clade *Hymenodictyon*+ Naucleaceae, though the reconstruction for the subtribes Mitragyninae and Corynantheinae is equivocal. The remaining nodes all reconstruct as possessing a protruding oncus.

4. Discussion

4.1. Pollen morphological characteristics

4.1.1. Size

Pollen grain size is rather uniform throughout the species investigated. The average equatorial diameter (E) varies from 9.01 μm in *Uncaria macrophylla* to 15.85 μm in *Cephalanthus tetrandra*. We found that *Cephalanthus* has the largest pollen grains in the tribe Naucleaceae, in agreement with Verellen et al. (2007).

4.1.2. Shape

In equatorial view, pollen shape is described by the P/E ratio. In the studied species, all of the pollen grains are spheroidal (P/E 0.88–1.14), or subprolate (P/E 1.14–1.33). The spheroidal condition can be further divided into oblate spheroidal (P/E 0.88–1.0) and prolate spheroidal (P/E 1.0–1.14). The amb is (sub)circular, and often somewhat lobed due to sunken colpi, which occur in most members of the Rubiaceae (Dessein et al., 2005a,b). Slightly triangular pollen grains are only found in *Neonauclea griffithii*.

4.1.3. Aperture

Number—The number of colpi is always three. Triaperturate pollen grains are common in the Rubiaceae, and are the plesiomorphic condition in the family (Dessein et al., 2005b). However, the number of apertures varies considerably within tribe Spermaceae (Dessein et al., 2002).

Position—In most Rubiaceae, pollen is angulaperturate (the apertures situated at the angles of the outline in polar view), or zonoaperturate (the apertures situated only at the equator) (Dessein et al., 2005b). All the species studied are zonoaperturate, the apertures

all arranged along the equator, but a tendency towards an angula-perture is seen in some pollen grains of *Neonauclea griffithii*.

Type—Apertures of Naucleaeae are always compound, built up by three components that are situated in three layers of the pollen wall. The aperture is built up of an ectocolpus, a mesoporus, and an endoaperture. This type of compound aperture is believed to be plesiomorphic in the Rubiaceae (Dessein et al., 2005b).

Ectocolpus—The length of the ectocolpus is very variable in the Rubiaceae (Dessein et al., 2005b). For instance, the relative length of the colpus (LC/P ratio) ranges from 6–62 in African *Spermacoce* species (Dessein et al., 2002). In the Naucleaeae, the variation in the LC/P ratio is relatively minor, ranging from 61 in *Uncaria sessilifructus* to 87 in *U. laevigata*. The apocolpium index is also very low, varying from 0.25 in *U. laevigata* to 0.53 in *Neonauclea sessilifolia*. The colpi are often slit-like in the genera *Uncaria*, *Metadina*, *Pertusadina*, and *Sinoadina*, sometimes becoming wider around the mesoporus. The width varies from 0.28 µm in *U. sessilifructus* to 2.29 µm in *Haldina cordifolia*. The ectocolpus membrane is often granular, except in *Uncaria hirsuta*, *U. macrophylla*, *U. rhynchophylloides*, *U. sessilifructus*, *Metadina trichotoma*, *Pertusadina hainanensis*, and *Sinoadina racemosa*. The ectocolpus membrane of these species is not visible, as the colpi are slit-like and deep-set.

The colpi of all species have distinct, regular margins. The ectocolpus is protuberant in *Cephalanthus tetrandra* and *Pertusadina hainanensis*.

Endoaperture—The variation in endoapertures in Rubiaceae is large and has significant systematic value. Endocolpi (Dessein et al., 2000) and endocinguli (Dessein et al., 2002) are the most common endoapertures in the family, but H-shaped endoapertures have also been reported in some taxa (Robbrecht, 1985; Huysmans et al., 1994; Verellen et al., 2007). H-shaped endoapertures have a thin H-shaped zone surrounding the porus. They can be clearly observed with SEM in broken pollen grains, while they appear as a brighter zone surrounding the porus in LM. The downstrokes of the “H” are parallel to the ectocolpus, while the equatorial connections may be weak or missing (Huysmans et al., 1994), making the “H” incomplete.

Distinct H-shaped endoapertures occur in *Neonauclea*, *Uncaria*, *Adina*, *Cephalanthus*, *Haldina*, *Metadina*, *Mitragyna*, *Sinoadina*, and *Hymenodictyon*, while indistinct ones occur in *Nauclea* and *Pertusadina*. H-shaped endoapertures are first observed here in *Adina*, *Cephalanthus*, *Metadina*, and *Sinoadina*. In *Haldina*, *Neonauclea*, *Sinoadina* and *Uncaria*, the “H” is incomplete. H-shaped endoapertures have also been previously reported in *Breonadia*, *Corynanthe*, *Haldina*, *Neonauclea*, *Pausinystalia*, *Pseudocinchona*, *Uncaria* (Verellen et al., 2007), and

Plate I. (see page 129)

SEMs of the Naucleaeae pollen.

Symbols: white arrowheads, protruding onci; white arrows, ectocolpi.

Scale bars: A–B, D, G–M, O=2 µm; C, F, N=1 µm; E=5 µm.

A. *Neonauclea griffithii*, polar view of pollen grain, with indistinct protruding oncus.

B–C. *N. sessilifolia*. B. Polar view of pollen grain. C. Detail of apocolpium, showing microreticulate sexine.

D. *N. truncata*, polar view of pollen grain.

E–F. *Uncaria hirsuta*. E. Polar view of pollen grain, showing protruding oncus. F. Detail of protruding oncus.

G. *U. laevigata*, equatorial view of pollen grain, showing protruding oncus.

H. *U. lancifolia*, equatorial view of pollen grain, with granular ectocolpus membrane.

I. *U. macrophylla*, polar view of pollen grain, showing sunken ectocolpus. This grain lacks a protruding oncus.

J. *U. rhynchophylla*, equatorial view of pollen grain, showing protruding oncus.

K. *U. rhynchophylloides*, equatorial view of pollen grain, with long, narrow ectocolpus.

L. *U. scandens*, equatorial view of pollen grain, with protruding oncus.

M–N. *U. sessilifructus*. M. Equatorial view of pollen grain, with slit-like ectocolpus. This grain lacks a protruding oncus. N. Detail of mesocolpium, showing striate-reticulate sexine.

O. *U. sinensis*, showing protruding oncus

Plate II. (see page 130)

SEMs of Naucleaeae and *Hymenodictyon* pollen.

Symbols: white arrowheads, protruding onci or remnants; black arrowheads, protuberances; white arrow, mesoporus.

Scale bars: A, E, G–M, O=2 µm; B, C=5 µm; D, F, N=1 µm.

A. *Adina pilulifera*, polar view of pollen grain, with protruding oncus.

B. *A. rubella*, polar view of pollen grain, showing protruding oncus.

C–D. *Cephalanthus tetrandra*. C. Oblique polar view of pollen grain, showing protuberance of ectocolpus (black arrowhead) and indistinct protruding oncus (white arrowhead). D. Detail of mesocolpium, showing perforate sexine.

E–F. *Haldina cordifolia*. E. Equatorial view of pollen grain, showing protruding oncus. F. Detail of mesocolpium, showing microreticulate sexine.

G. *Metadina trichotoma*, equatorial view of pollen grain, showing protruding oncus.

H. *Mitragyna rotundifolia*, equatorial view of pollen grain.

I. *Nauclea officinalis*, oblique polar view of pollen grain, showing protruding oncus.

J. *Neolamarckia cadamba*, polar view of pollen grain, showing protruding oncus.

K. *Pertusadina hainanensis*, equatorial view of pollen grain, showing protuberance of ectocolpus. This grain lacks a protruding oncus.

L. *Sinoadina racemosa*, polar view of pollen grain, show protruding oncus remnants.

M–N. *Hymenodictyon flaccidum*. M. Oblique polar view of pollen grain. N. Equatorial view of pollen grain, showing protruding oncus remnants.

O. *H. orixense*, equatorial view of pollen grain, showing mesoporus.

Plate IV. (see page 132)

TEMs of Naucleaeae pollen.

Abbreviations: c, columellae; co, costa; en, endexine; fl, foot layer; in, intine; lm, lipidic material; n, nexine; re, remnant; t, tectum; v, vacuole. Symbols: black arrowheads, electron-dense outer layer of oncus; star, electron-lucent inner layer of oncus.

Scale bars: A, E–F, H–I=200 nm; B, D, J=500 nm; C, G=100 nm.

A–B. *Adina pilulifera*. A. Pollen wall ultrastructure in non-apertural region. B. Detail of pollen wall structure towards apertural region, showing protruding oncus remnant and costae.

C–D. *Metadina trichotoma*. C. Pollen wall ultrastructure in non-apertural region, and possible lipidic material between columellae. D. Detail of pollen wall structure towards apertural region.

E–F. *Neolamarckia cadamba*. E. Detail of pollen wall stratification in non-apertural region. Note possible lipidic material between columellae. F. Oncus separated from the cytoplasm of the vegetative cell by intine material.

G–H. *Pertusadina hainanensis*. G. Detail of pollen wall structure in non-apertural region. Note possible lipidic material between columellae. H. Protruding oncus and costae in apertural region.

I–J. *Uncaria hirsuta*. I. Detail of pollen wall stratification in non-apertural region. J. Protruding oncus and costae in apertural region. Note the vacuole in oncus.

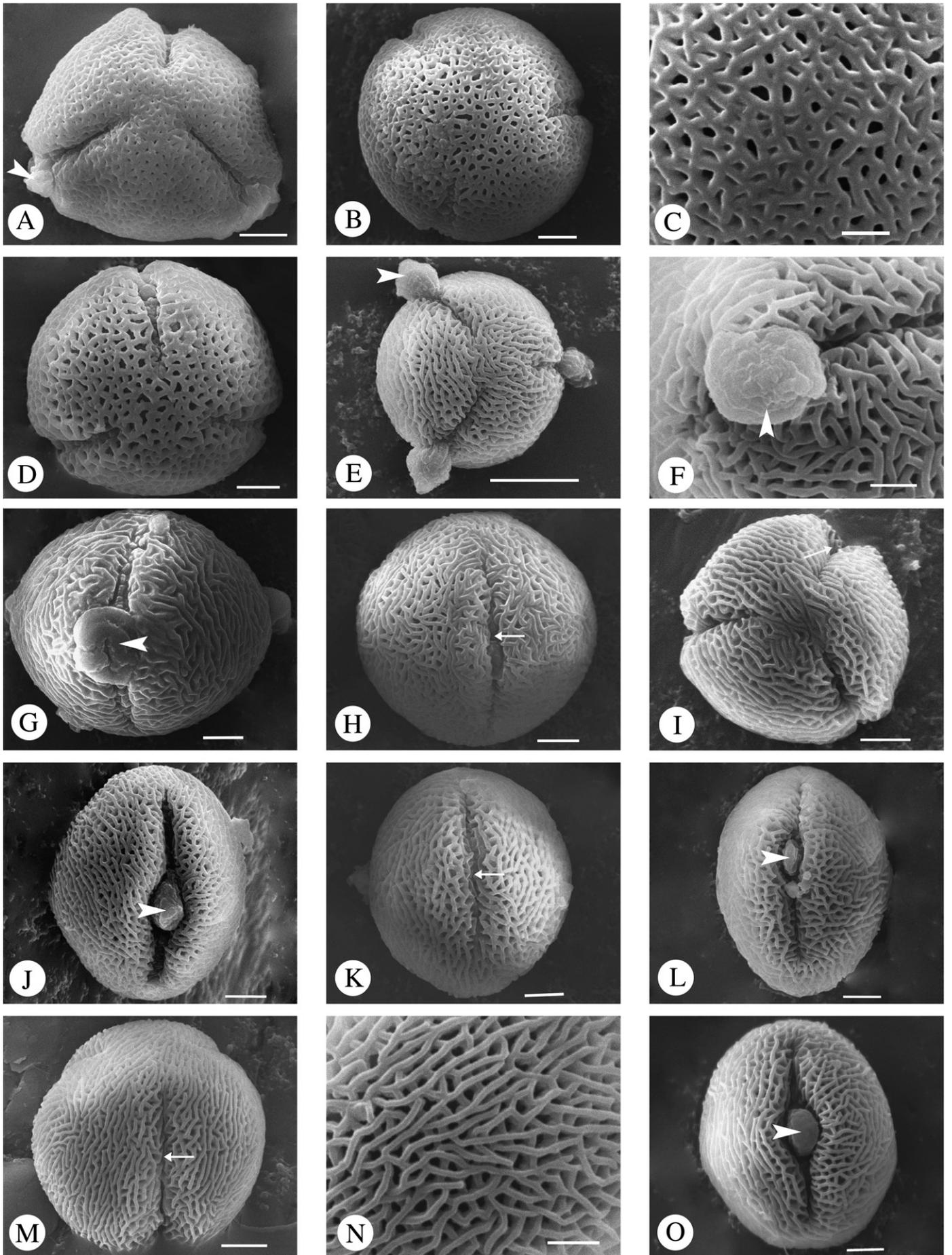


Plate I. (caption on page 128).

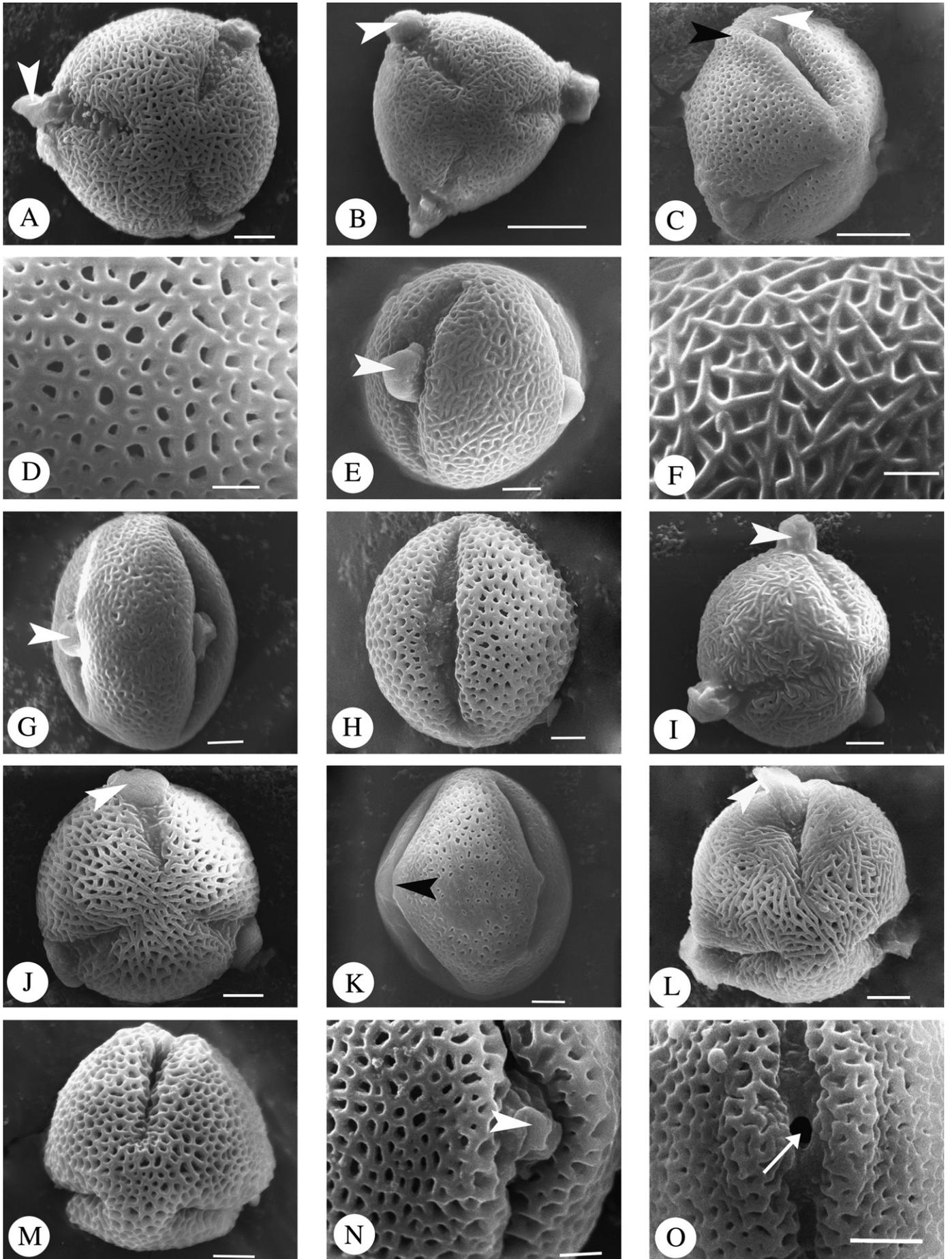


Plate II. (caption on page 128).

Mitragyna s.l. (Huysmans et al., 1994). The endoaperture is indistinct in *Myrmeconuclea*, and alongate in *Sarcocephalus* (Verellen et al., 2007). In the remaining seven genera of the Naucleae the characteristics of the endoaperture remain unknown.

4.1.4. Sexine ornamentation

There is little variation in sexine patterns in the Naucleae. The sexine ornamentation of most species is microreticulate to striate, rugulate, or perforate. In this investigation, only *Cephalanthus tetrandra*

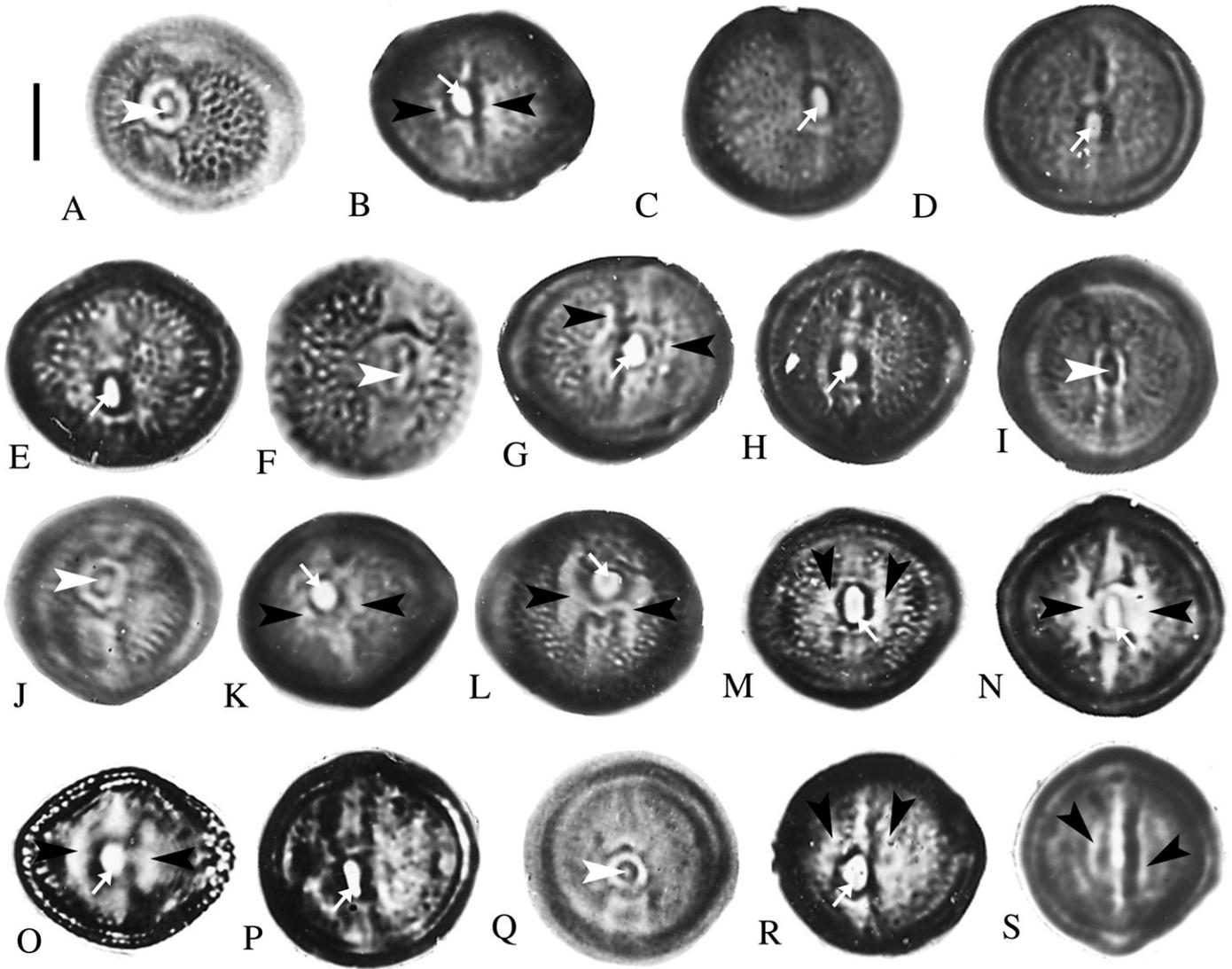


Plate III.

Light micrographs of Naucleae and *Hymenodictyon* pollen in equatorial view.

Symbols: white arrowheads, protruding onci visible as two concentric circles over the mesopori; black arrowheads, H-shaped endoapertures visible as slightly brighter zones surrounding the mesopori; white arrows, mesopori.

Scale bar = 5 μ m.

- A. *Neonauclea griffithii*, showing a protruding oncus in the center of the mesopori.
- B. *N. sessilifolia*, with incompletely H-shaped endoaperture and alongate mesopori.
- C. *N. truncata*, showing alongate mesopori.
- D. *Uncaria hirsuta*, with alongate mesopori. This grain lacks a protruding oncus.
- E. *U. lancifolia*, with alongate mesopori.
- F. *U. macrophylla*, showing indistinct protruding oncus in the center of the mesopori.
- G. *U. rhynchophylla*, with indistinctly H-shaped endoaperture and subcircular mesopori. This grain lacks a protruding oncus.
- H. *U. scandens*, showing subcircular mesopori. This grain lacks a protruding oncus.
- I. *U. sessilifrutus*, showing a protruding oncus in the center of the mesopori.
- J. *U. sinensis*, with a protruding oncus in the center of the mesopori.
- K. *Adina pilulifera*, with indistinctly H-shaped endoaperture and circular mesopori. This grain lacks a protruding oncus.
- L. *Cephalanthus tetrandra*, with circular mesopori and distinct H-shaped endoaperture. This grain lacks a protruding oncus.
- M. *Haldina cordifolia*, with alongate mesopori and incompletely H-shaped endoaperture. This grain lacks a protruding oncus.
- N. *Metadina trichotoma*, with alongate mesopori and distinctly H-shaped endoaperture. This grain lacks a protruding oncus.
- O. *Mitragyna rotundifolia*, with circular mesopori and distinct H-shaped endoaperture.
- P. *Nauclea officinalis*, with alongate mesopori. This grain lacks a protruding oncus.
- Q. *Pertusadina hainanensis*, with a protruding oncus in the center of mesopori.
- R. *Sinoadina racemosa*, with indistinctly H-shaped endoaperture and subcircular mesopori. This grain lacks a protruding oncus.
- S. *Hymenodictyon flaccidum*, with H-shaped endoaperture. This grain lacks a protruding oncus.

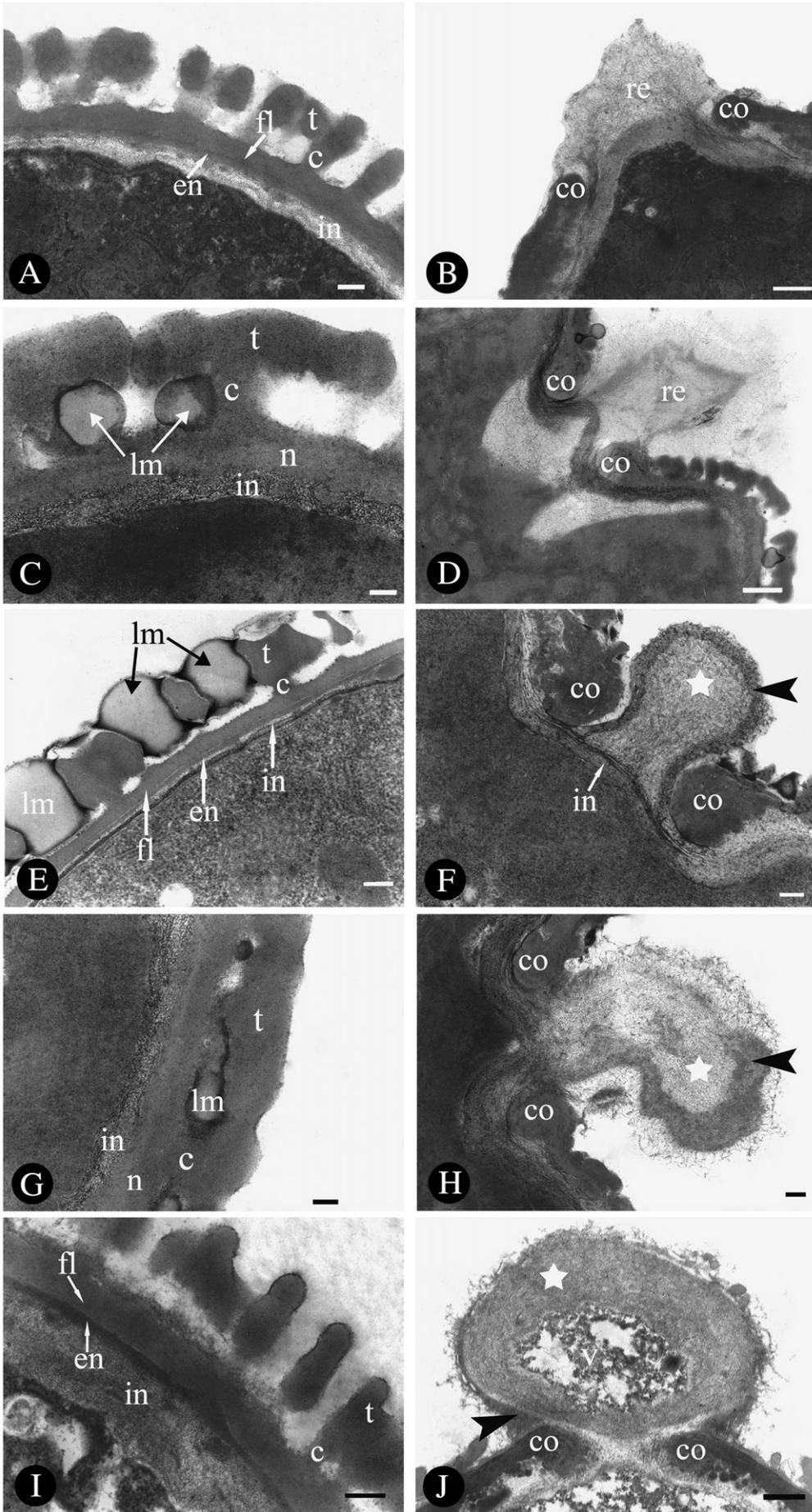


Plate IV. (caption on page 128).

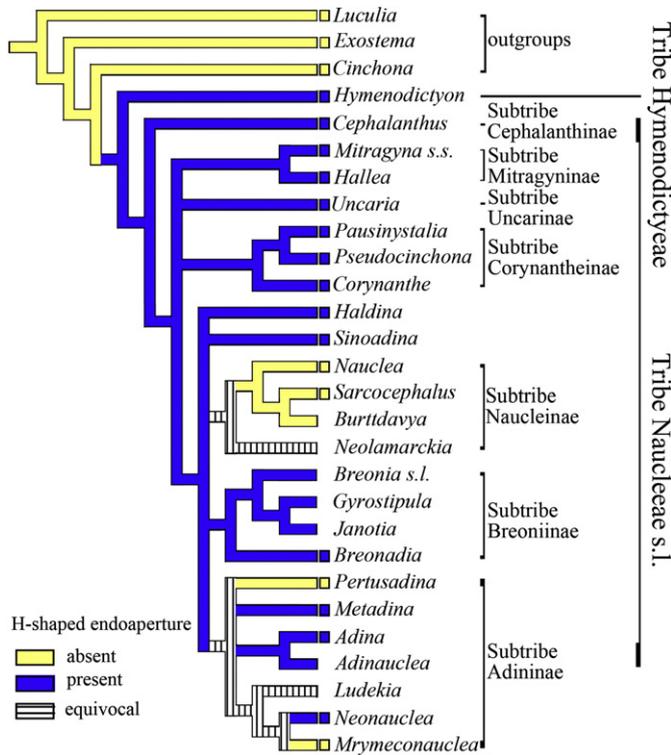


Fig. 1. Phylogeny of Naucleaeae-Hymenodictyaceae from Razafimandimbison and Bremer (2001, 2002) with H-shaped endoapertures mapped and the ancestral states reconstructed. A box (yellow or blue) following a taxon name reports the state of H-shaped endoaperture in the taxon. The absence of a box means data is missing for this taxon. Yellow is the primitive state, viz. H-shaped endoaperture absent, blue the advanced, viz. H-shaped endoaperture present.

has a perforate sexine. The other species have microreticulate to striate, or slightly rugulate sexines. The lumina/perforations of the microreticulations are smaller than 1 μm in diameter, and are usually irregularly polygonal or (sub)circular. There is usually no differentiation of the sexine towards the poles and/or colpi. The sub-parallel muri are cross-linked to form a reticulum, with the connections between the muri lying either on a single level, or different levels. The muri surface is usually clean. Supratracteal elements are absent, as in the majority of Rubiaceae (Dessein et al., 2005b).

4.1.5. Protruding oncus

The term “oncus” was first proposed by Hyde (1955) to describe a lens-shaped thickening of the intine occurring beneath the apertures. Ramam (1954), and Farooq and Inamuddin (1969) found that the intine thickenings often protruded through the apertures forming papillae in the Rubiaceae. Philip and Mathew (1975) introduced the term “pollen bud” to describe these papillae, when they contained cytoplasmic contents.

Weber (1996) proposed that the apertural intine protrusions be divided into two types: (1) those in which the protrusions are separated from the cytoplasm of the vegetative cell by intine material, and are eliminated from the grains before shedding (Igersheim and Weber, 1993; Weber and Igersheim, 1994); (2) those in which the protrusions contain cytoplasmic material, and remain attached to the pollen grains (Sniezko and Bell, 1985; Takahashi and Skvarla, 1990; Noher de Halac et al., 1992). Both types are found in the Rubiaceae (Igersheim, 1993; Weber, 1996). Tilney and Van Wyk (1997) proposed the new term “protruding oncus” as an alternative to pollen bud, but did not distinguish between structures that contain cytoplasmic contents and those that do not. Hansson and El-Ghazaly (2000) proposed a slightly different division into two types: (A) the protruding oncus alone (papillae); (B) a protruding oncus containing cytoplasmic contents, which separates from the pollen grain before

shedding. Although the types of protruding oncus proposed by Hansson and El-Ghazaly (2000) differ from Weber’s (1996), both definitions focus on whether or not the onci contain cytoplasm, and whether or not they are eliminated from the grains before shedding.

In this study, the protruding onci become separated from the pollen grains leaving shapeless remnants in *Adina*, *Cephalanthus*, *Metadina*, *Pertusadina*, *Sinoadina*, and *Hymenodictyon*. The onci remain attached to the pollen grains in *Neonauclea*, *Uncaria*, *Haldina*, *Nauclea*, and *Neolamarckia*. Cytoplasmic contents were found in the onci of *Uncaria hirsuta*, but not in all grains. This suggests that the presence or absence of cytoplasmic contents is not a valid character for distinguishing pollen buds from protruding onci, or for distinguishing types of protruding onci. No cytoplasmic contents were found in any grains of *Adina pilulifera*, *Metadina trichotoma*, *Neolamarckia cadamba*, or *Pertusadina hainanensis*.

Protruding onci were not reported by Verellen et al. (2007) in their investigation of Naucleaeae pollen. According to Punt et al. (2007), onci are not resistant to standard pollen acetolysis, a fact confirmed by a controlled experiment carried out as part of this investigation. Unlike the present investigation in which acetolysis was only carried out for 3–5 min, Verellen et al. (2007) prepared their pollen for 10 min, which may have destroyed the intine protrusions.

4.1.6. Pollen wall stratification

The exine is composed of a discontinuous tectum, a row of columellae, and a nexine layer. This pattern corresponds to the basic pattern of pollen-wall stratification in angiosperms (Dessein et al., 2005b). The nexine is often differentiated into a foot layer and an endexine. The nexine bordering the ectocolpus is often thickened into costae. In the mesocolpium, nexine thickness varies between 0.06–0.35 μm, the columellae thickness between 0.01–0.25 μm, and the tectum thickness between 0.16–0.48 μm. The tectum is usually the thickest layer of the pollen wall.

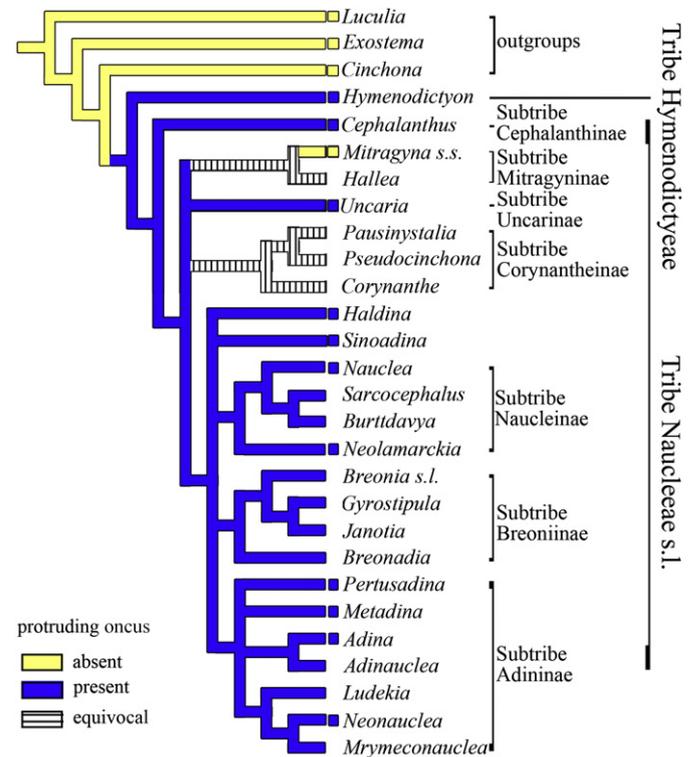


Fig. 2. Phylogeny of Naucleaeae-Hymenodictyaceae from Razafimandimbison and Bremer (2001, 2002) with protruding onci mapped and the ancestral states reconstructed. A box (yellow or blue) following a taxon name reports the state of the protruding oncus in the taxon. The absence of a box means data is missing for this taxon. Yellow is the primitive state, viz. protruding oncus absent, blue the advanced, viz. protruding oncus present.

Nexine—The nexine may or may not appear differentiated into a foot layer and an endexine. In this study, the nexine which appears differentiated is only found in taxa that were investigated from fresh grains (*Adina pilulifera*, *Neolamarckia cadamba*, *Uncaria hirsuta*). The nexine, which does not appear differentiated into a foot layer and an endexine occurs in *Metadina trichotoma* and *Pertusadina hainanensis*, taxa whose pollen was collected from herbarium sheets. The absence of a foot layer may thus be an artifact arising from the source of the pollen.

Columellae—Columellae are well developed in *Adina pilulifera*, *Metadina trichotoma*, and *Uncaria hirsuta*, but indistinct in *Neolamarckia cadamba* and *Pertusadina hainanensis*. The columellae of the mesocolpia are obviously thicker than that immediately surrounding the colpi, especially in *A. pilulifera* and *U. hirsuta*. The space between the columellae is wider than the transverse thickness of columellae in four species (*A. pilulifera*, *M. trichotoma*, *N. cadamba* and *U. hirsuta*). In *P. hainanensis* it is smaller. There are no processes between the columellae in *A. pilulifera* and *U. hirsuta*, but there are potentially lipidic materials between columellae in *N. cadamba*, *M. trichotoma*, and *P. hainanensis*. They are especially large in *N. cadamba*.

Intine—Intine thickness varies from 0.01–1.03 μm . The intine in the mesocolpus is of approximately the average thickness, but is thickened into a bubble-like protruding oncus above the mesoporus.

4.1.7. Ultrastructure of the protruding oncus

In *Adina pilulifera*, *Metadina trichotoma*, and *Pertusadina hainanensis* only the remnants of the oncus are visible. In *Uncaria hirsuta* and *Neolamarckia cadamba*, the oncus is subcircular and protrudes substantially from the aperture with a head and neck structure. The oncus wall is composed of a bi-layered ectintine with an electron-dense outer layer, and an electron-lucent inner layer. In *U. hirsuta*, there is cytoplasmic material in the center of the oncus.

4.2. Systematic significance of palynological characters

4.2.1. General pollen morphology

Plants of the Naucleaeae have preserved many plesiomorphic features in pollen morphology, e.g., aperture morphology and the pollen wall structure. There is little variation in pollen size, shape, pollen wall stratification, or number and type of aperture in the studied species. Verellen et al. (2007) considered the Naucleaeae to be fairly stenopalynous, since pollen morphology was similar in all of the species included in their investigation. Their palynological evidence supports the broader delimitation of the Naucleaeae *sensu* Razafim. and Bremer, but cannot provide unambiguous support for subtribal or generic delimitations because of a lack of variation in pollen characters. Our palynological results agree well with those of Verellen et al. (2007).

The pollen morphology of tribe Hymenodictyaeae is very similar to that of the Naucleaeae. Razafimandimbison and Bremer (2001) placed the Hymenodictyaeae as sister to the Naucleaeae. Based on this result, Andersson and Antonelli (2005) submerged the Hymenodictyon into the Naucleaeae for molecular analysis. Razafimandimbison and Bremer (2006) showed that the two tribes can still be distinguished from each other by their inflorescence morphology, floral disk morphology, and fruit characteristics. Verellen et al. (2007) concluded that the sister relationship between the two tribes was supported by the shared presence of H-shaped endoapertures, but they also noted that their palynological evidence also supported the submersion of Hymenodictyaeae in Naucleaeae. H-shaped endoapertures, and a protruding oncus, occur in at least some members of the Hymenodictyaeae and Naucleaeae included in this paper, and their pollen morphology in other respects also shows much similarity (e.g., sexine ornamentation, aperture morphology). The lack of clear synapomorphies for the two tribes, and the shared presence of H-shaped endoapertures and a protruding oncus suggest that Anderson and Antonelli's (2005) analysis may be correct.

4.2.2. H-shaped endoaperture

The presence of H-shaped endoapertures was proposed as a synapomorphy of the Naucleaeae by Dessein et al. (2005b), and Verellen et al. (2007) agreed. To test this hypothesis, we mapped the occurrence of H-shaped endoapertures at the generic level on the phylogenetic tree of Razafimandimbison and Bremer (2001, 2002), and reconstructed the ancestral states at each node. H-shaped endoapertures are widespread in five subtribes of the Naucleaeae, and in the Hymenodictyaeae, and form a putative synapomorphy of the clade *Hymenodictyon*+ Naucleaeae.

4.2.3. Protruding oncus

The protruding oncus was previously reported in the following Rubiaceae taxa: *Stephegyne parviflora* (Ramam, 1954), *Oldenlandia nudicaulis* (Farooq and Inamuddin, 1969), Ophiorrhizaceae (Philip and Mathew, 1975; Mathew and Philip, 1987), Isertieae (Priyadarshan and Ramachandran, 1984), and Vanguerieae (Tilney and Van Wyk, 1997), *Mitriostigma axillare* (Hansson and El-Ghazaly, 2000), Hedyotideae (Ma et al., 2005), and *Tarenna gracilipes* (Vinckier and Smets, 2005). These taxa are not closely related according to the summary cladogram of Rubiaceae (Dessein et al., 2005b), which suggests that the character has evolved several times independently.

In the Hymenodictyaeae-Naucleaeae the presence of the protruding oncus has only been investigated in 12 genera. Of the seven subtribes, only the Mitragyninae probably lack it. The Adininae, Naucleinae, Uncarinae, and Cephalanthinae all possess this characteristic, which implies a relatively close relationship between these four subtribes. The condition in the Corynantheinae and Breoniinae remains unknown, though parsimony analysis suggests that the Breoniinae will be found to possess it. The sister tribe Hymenodictyaeae possesses a protruding oncus, while the outgroups lack it. These results lead us to suggest that the protruding oncus is a synapomorphy of the Hymenodictyaeae-Naucleaeae clade. The generic-level ancestral state reconstruction supports this suggestion.

5. Conclusions

The Naucleaeae is a stenopalynous tribe, characterized by very small to small pollen grains, tricolporate pollen with an ectocolpus, a subcircular to lolongate mesoporus, and often an H-shaped endoaperture. Sexine patterns are microreticulate to striate, rugulate, or perforate. Our pollen morphological observations in Chinese Naucleaeae species support the delimitation of the Naucleaeae *sensu* Razafim. and Bremer. Naucleaeae have preserved many plesiomorphic features in pollen morphology, and pollen morphology is of little value in distinguishing the subtribes and genera of the Naucleaeae. Ancestral state reconstruction at the generic level is unambiguous in showing that the possession of H-shaped endoapertures and protruding onci form morphological synapomorphies of the clade *Hymenodictyon*+ Naucleaeae.

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