

The morphology and exine ornamentation of fresh pollen from four dipterocarp species in Sabah

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Summary. The pollen morphology and fine exine ornamentation of the dipterocarps *Parashorea tomentella* (Symington) Meijer, *Shorea multiflora* (Burck) Symington, *Shorea xanthophylla* Symington and *Shorea leprosula* Miq. were investigated from individuals growing in Sepilok Forest Reserve, Sabah. Fresh pollen was extracted from flowers collected in the canopy and analyzed using a combination of light microscopy and Scanning Electron Microscopy (SEM). The size of pollen grains was estimated from their polar axis length and equatorial diameter, and systematic descriptions were developed from the SEM images. The four species had contrasting polar axis lengths and equatorial diameter dimensions that varied in the range 11.7–38.0 μm and 11.5–32.7 μm , respectively. The pollen was monad, tricolpate and predominately spheroidal but the morphology varied among the species. *Parashorea tomentella* had narrow colpi and wide irregular muri. *Shorea multiflora* and *S. xanthophylla* had colpi covered by a colpus membrane with a microreticulate ornamentation. Pollen of *S. leprosula* were prolate spheroidal, subspheroidal or subprolate, lobate in polar view, with two obvious colpi and a microreticulate ornamentation. For these four species, pollen volume was significantly correlated to flower size. We conclude that with more research, pollen morphology could be a diagnostic tool for distinguishing among dipterocarp species in different sections of a genus (e.g. sections *Mutica* and *Richetioides* of *Shorea*) and between genera (*Shorea* and *Parashorea*). However, in our study, the two species of *Shorea* section *Richetioides* could not be distinguished on the basis of morphological differences.

Angiosperms exhibit a great diversity in pollen exine ornamentation and morphology. These traits can be used as a diagnostic tool for identifying plant families and species in studies of reproductive biology, palaeoecology and forensic science (Cruden & Lyon, 1985). Pollen identification is also a valuable technique for confirming the identity of pollen carried by flower visitors, and may contribute to investigations of pollination ecology and pollinator dynamics. The aim of this paper is to document previously undescribed information and images on pollen morphology and fine ornamentation of four dipterocarp species as an aid to our research on their reproductive ecology and pollination biology (Maycock *et al.*, 2008).

There are few published descriptions of the pollen of the Dipterocarpaceae. Investigations were reviewed by Ashton (1988) but the most comprehensive studies that include systematic descriptions and high resolution images are by Maury *et al.* (1975) and Talip (2008). Maury *et al.* (1975) documented pollen descriptions for nine species from six genera of Dipterocarpaceae and summarised existing information on another 26 species, while Talip (2008) examined the pollen morphology of 32 species of Malaysian dipterocarps. However, these studies were both based on pollen gathered from herbarium specimens, and there are concerns that drying may distort their shape and outline (Price & Ayers, 2008). Price and Ayers (2008) reported that the shape of pollen may change from oblate spheroidal to subprolate as a result of drying time and temperature, and recommended that either FAA-preserved material collected in the field, or fresh pollen, should be used for morphological studies.

The value of previous pollen descriptions have not been fully realised in research on dipterocarp reproductive biology and pollination ecology. To our knowledge, there are no studies of dipterocarp pollinators that have determined pollen loads using high resolution images. These images are important for robust identifications of pollen and pollinator, particularly when the pollen load contains a mixture of species, and for quantifying pollen load. There are also very few images and systematic descriptions of the pollen of dipterocarps in Sabah, and very little association between existing material and dipterocarp pollination ecology.

The aims of this study were (a) to describe pollen morphology based on freshly collected pollen of four dipterocarp species that are the subject of an on-going study of pollination biology, (b) to identify the extent of variation in pollen morphology between the four species, (c) to present clear high resolution images of the pollen as a reference for future taxonomic and ecological research, and (d) to determine the interspecific differences in pollen size between these four species and relate these differences to flower size.

Materials and Methods

The species studied were *Parashorea tomentella* (Symington) Meijer, *Shorea multiflora* (Burck) Symington, *Shorea xanthophylla* Symington and *Shorea leprosula* Miq. The fresh

flowers of *P. tomentella* are larger (mean calyx diameter 4.2 mm) than those of *S. leprosula* (mean calyx diameter 2.2 mm), and *S. multiflora* and *S. xanthophylla* (mean calyx diameter 1.3 mm and 1.2 mm, respectively). Previous research has indicated that the pollen from the Asiatic sub-family of the Dipterocarpaceae (Dipterocarpoideae) is monad, tricolpate and predominately spherical, while colpi are generally elongate and narrow, and covered with a thin colpus membrane. The exine consists of a thin endexine layer and a stronger ectexine layer (Maury *et al.*, 1975).

Pollen was collected during a minor dipterocarp flowering event in the Kabili-Sepilok Forest Reserve, in Sabah, Malaysia (5°10' N, 117°56' E) between May and July 2007. Pollen was sampled from fresh flowers that were collected during anthesis using mixed rope techniques. Inflorescences were removed by hand and placed in sealed bags. Flowers were dissected using a dissecting microscope and the pollen was carefully removed using drops of 70% ethanol. All ethanol residues were collected on a glass slide and the slide was analysed systematically; any clusters of pollen were carefully dispersed using a fine needle. Only fresh pollen was used for analysis and imaging. The analysis of pollen followed techniques described by Dafni (1992) and Shivanna (2003). Initial investigations of pollen morphology were conducted using light microscopy (LM) and a digital microscope imaging device (DMID), using the software SemAfore (JEOL, Sweden). The polar axis length and equatorial diameter of 50 individual pollen grains of each species were measured using the DMID.

For scanning electron microscopy (SEM), pollen grains were fixed in 70% ethanol and dehydrated in 100% ethanol, then transferred to hexamethyldisilazane for five minutes and allowed to air dry. Dehydrated pollen grains were then mounted onto metal stubs using double-sided adhesive tabs, sputter coated with gold using a EMitech K550 Sputter Coater (Quorum Technologies Ltd, UK), and examined using a JEOL 35CF Scanning Electron Microscope (JEOL, Japan) at an accelerating voltage of 10 kV and at different degrees of magnification. Dehydration and the vacuum created in the SEM chamber can cause pollen grains to distort (Hesse *et al.*, 2009). Therefore SEM images were compared with images obtained using the DMID in order to verify that the systematic descriptions of morphology and fine ornamentation were not biased by these distortions. Images were manipulated using the software Adobe Photoshop CS2 (Adobe, USA) and then compiled into an electronic reference library. Morphological descriptions of the pollen were created based on the SEM images, light microscopy and Punt *et al.* (2007). The following abbreviations are used for the pollen descriptions: P/E = Polar axis/Equatorial diameter, P = Polar axis, E = Equatorial diameter, PLL = Plica length, PLW = Plica width, CL = Colpus length, CW = Colpus width, L = Lumina length, M = Murus diameter.

The Kruskal-Wallis χ^2 was used to test for differences among species in polar axis length and equatorial diameter. We determined the volume of the pollen for each species using the formula $\pi PE^2/6$ (Harder, 1998). We also estimated pollen volume using mean polar length

and equatorial diameter data from Talip (2008) for eight species that occur within the Sepilok Forest Reserve. From previous studies (Maury *et al.*, 1975; Talip, 2008), it is apparent that there is significant inter-specific variation in pollen size and this is thought to be related to flower size and nutrient supply to the developing bud (Müller, 1979 cited in Talip, 2008), so we used linear models to investigate the relationship between flower size and pollen volume for our data and those of Talip (2008).

Results and Discussion

Systematic descriptions of pollen

Parashorea tomentella

The pollen was the largest of the four species (Fig. 1, P/E: $26.6 \pm 0.4 \times 25.9 \pm 0.4 \mu\text{m}$, $n = 50$). Individual grains were spheroidal, subspheroidal, oblate spheroidal or prolate

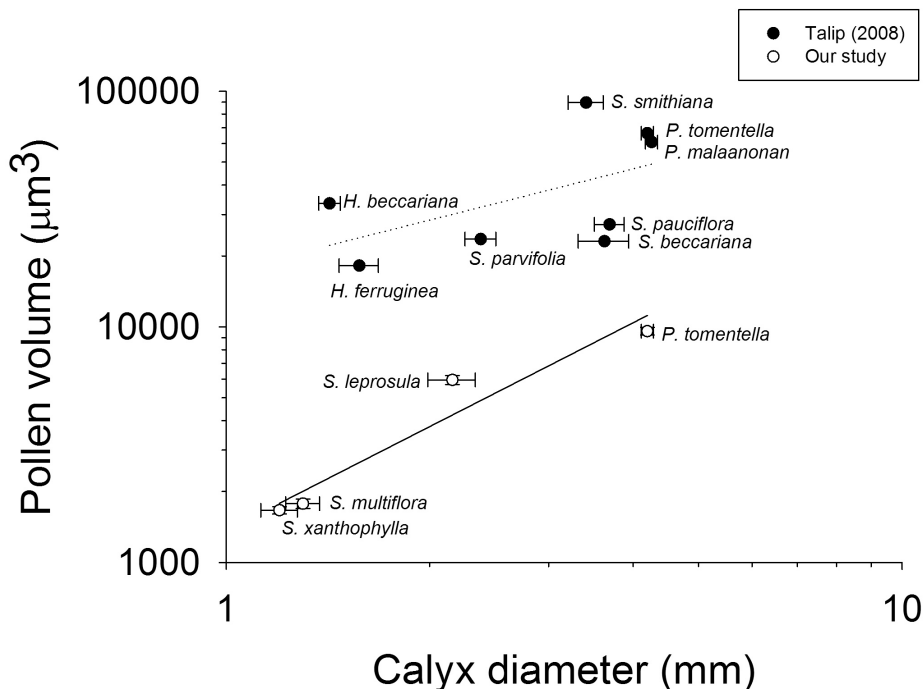


Fig. 1. Relationship between flower size, defined as the calyx diameter of the open flower, and pollen volume for the four species of dipterocarp sampled in Sepilok Forest Reserve, Sabah, Malaysia, and eight of the species examined by Talip (2008). The lines indicate the linear models for these data; the solid line indicates a significant relationship ($p < 0.05$) and a dotted line a non-significant relationship. The apparent difference in pollen volume between the two provenances of *P. tomentella* is discussed in the text.

spheroidal ($P/E = 0.98 \pm 0.02$), tricolpate, with narrow long colpi (CW: $1.6 \mu\text{m} \pm 0.5$, $n = 4$ colpi on four pollen grains, CL: $20.9 \mu\text{m} \pm 1.0 \mu\text{m}$, $n = 4$ colpi on three pollen grains). From LM images, we observed that one colpus was deeper and wider than the other two. SEM images suggested that one colpus culminated in an ectoaperture on one polar field (Fig. 2a & 2b). Light microscopy suggested that there was a thickening of the intine layer or a delimiting of the mesocolpium. The exine was microreticulate (Fig. 2c) with crotonoid patterning and wide muri (M: $0.62 \pm 0.03 \mu\text{m}$, $n = 10$ muri on one pollen grain). Segments of the muri were triangular and rectangular. The shape and dimensions of the lumina ranged from narrow and elongate to irregular and spherical (L: $1.03 \pm 0.12 \mu\text{m}$, $n = 10$ lumina on one pollen grain).

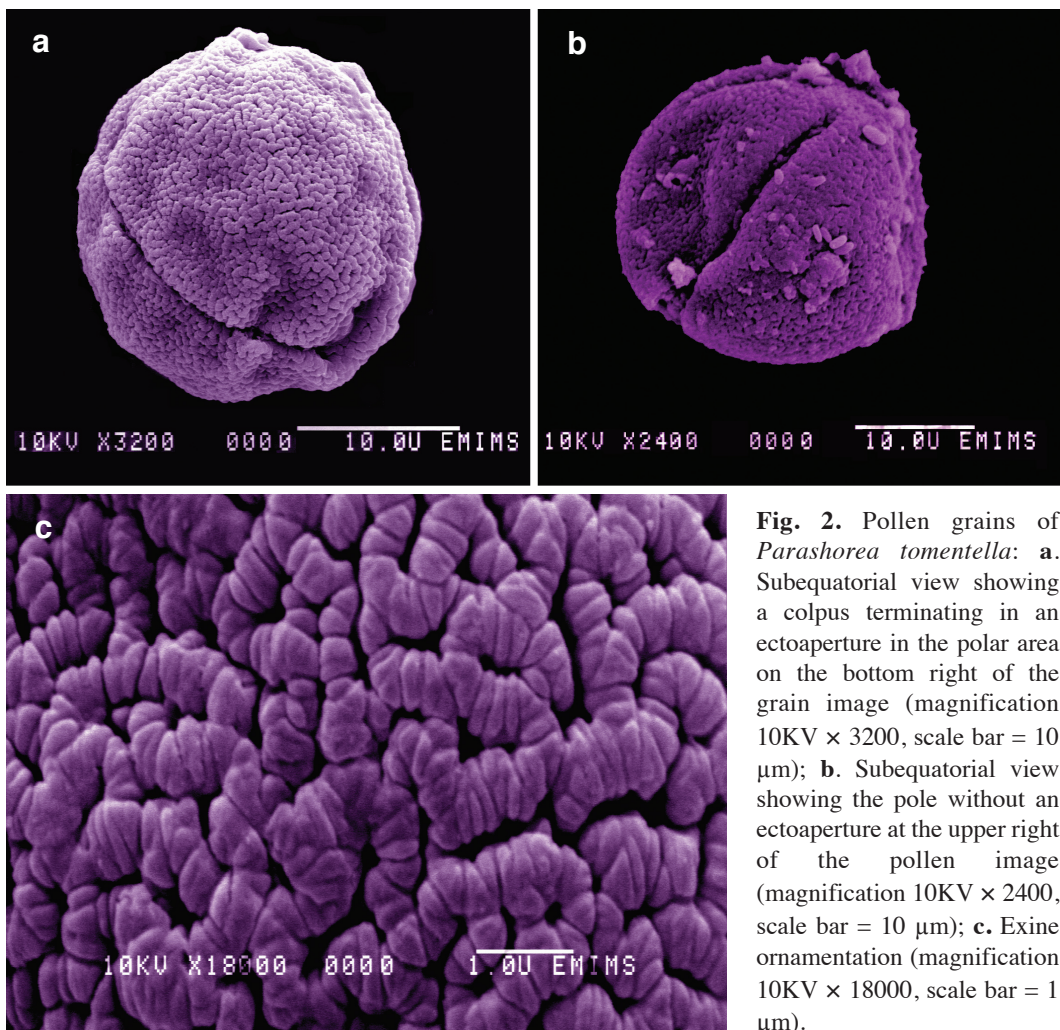


Fig. 2. Pollen grains of *Parashorea tomentella*: **a.** Subequatorial view showing a colpus terminating in an ectoaperture in the polar area on the bottom right of the grain image (magnification 10KV \times 3200, scale bar = 10 μm); **b.** Subequatorial view showing the pole without an ectoaperture at the upper right of the pollen image (magnification 10KV \times 2400, scale bar = 10 μm); **c.** Exine ornamentation (magnification 10KV \times 18000, scale bar = 1 μm).

Shorea multiflora

The pollen grains were small (P/E: $16.0 \pm 0.3 \mu\text{m} \times 14.4 \pm 0.2$, $n = 50$). Individual grains were spheroidal, subspheroidal or oblate spheroidal (P/E = 0.91 ± 0.02), tricolpate with three ectocolpi that were covered in a colpus membrane (Fig. 3a & 3b). The colpi were moderately long (CL: $10.1 \pm 0.4 \mu\text{m}$, $n = 2$ colpi on two pollen grains) but narrow (CW: $2.6 \pm 0.8 \mu\text{m}$, $n = 6$ colpi on four pollen grains). The polar fields were broad and the distances between the apices of the colpi were approximately equal (average $5.7 \mu\text{m} \pm 0.1$, $n = 3$). Light microscope images suggested that the endexine layer was thicker to support the exine layer between the colpi. The exine ornamentation was microreticulate, with a compact croton pattern. The raised muri (M: $0.23 \pm 0.01 \mu\text{m}$, $n = 10$ muri on one pollen grain) were scored into segments, primarily triangular but also rectangular (Fig. 3c). The microreticulate lumina were spherical to elliptical (L: $0.14 \pm 0.01 \mu\text{m}$, $n = 10$ lumina on one pollen grain) and isodiametric.

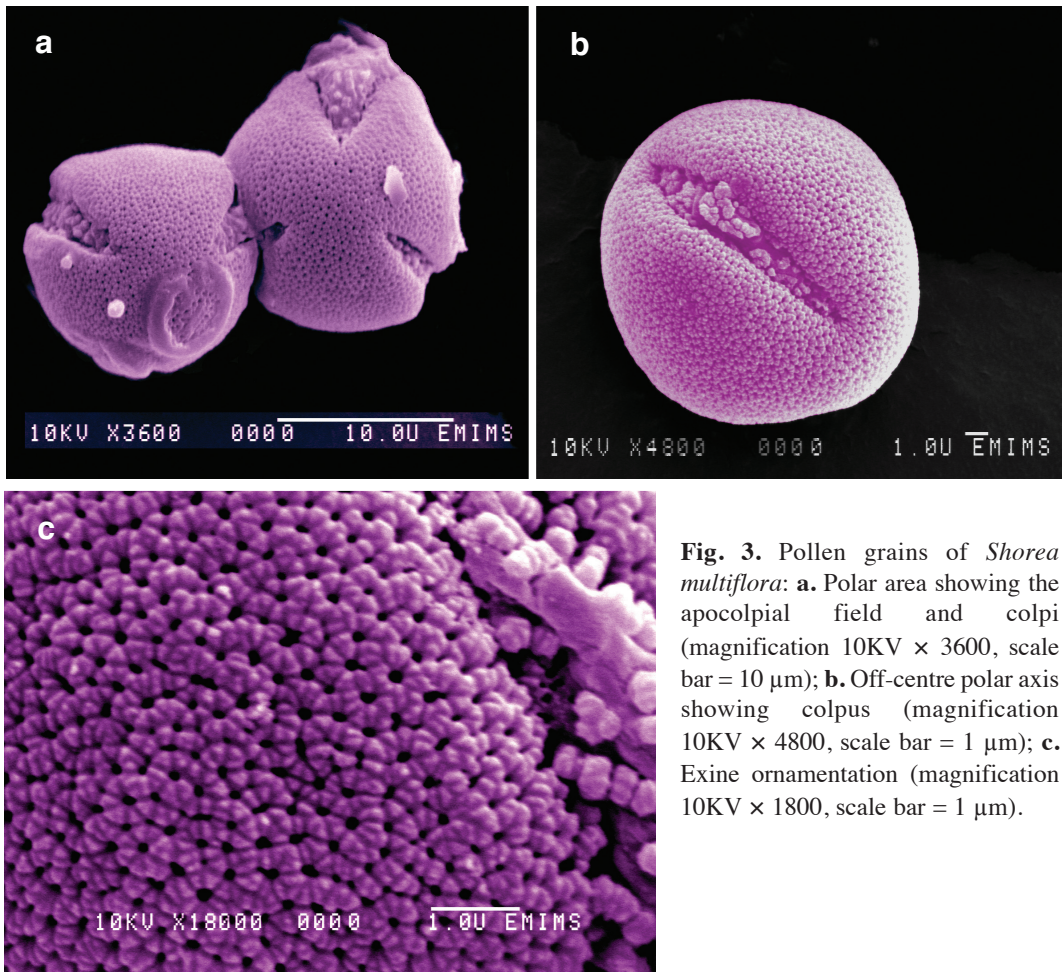


Fig. 3. Pollen grains of *Shorea multiflora*: **a.** Polar area showing the apocolpial field and colpi (magnification 10KV \times 3600, scale bar = 10 μm); **b.** Off-centre polar axis showing colpus (magnification 10KV \times 4800, scale bar = 1 μm); **c.** Exine ornamentation (magnification 10KV \times 1800, scale bar = 1 μm).

Shorea xanthophylla

The structure, size and shape of *S. xanthophylla* pollen were similar to those of *S. multiflora*. The pollen grains were small (P/E: $14.7 \pm 0.2 \times 14.6 \pm 0.2 \mu\text{m}$, $n = 50$), spheroidal, subspheroidal or oblate spheroidal (P/E = 1.00 ± 0.02) and tricolpate (Fig. 4a & 4b). The three colpi were narrower than *S. multiflora* (CW: $1.1 \pm 0.3 \mu\text{m}$, $n = 3$ colpi on two pollen grains) and covered with a colpus membrane. The orientation of the pollen grain in the SEM image prevented us from recording multiple colpi lengths (CL: $9.1 \mu\text{m}$, $n = 1$ colpus). The apices of the colpi were approximately equal in dimension (average $4.2 \mu\text{m} \pm 2.5$, $n = 3$ apices on one pollen grain). The exine ornamentation was microreticulate, with a compact croton pattern. The raised muri (M: $0.24 \pm 0.01 \mu\text{m}$, $n = 10$ muri on one pollen grain) were also scored into segments that were primarily triangular but also rectangular (Fig. 4c). Lumina were spherical to elliptical, and approximately isodiametric, although there was substantial variation in lumen size (L: $0.16 \pm 0.01 \mu\text{m}$, $n = 10$ lumina on one pollen grain).

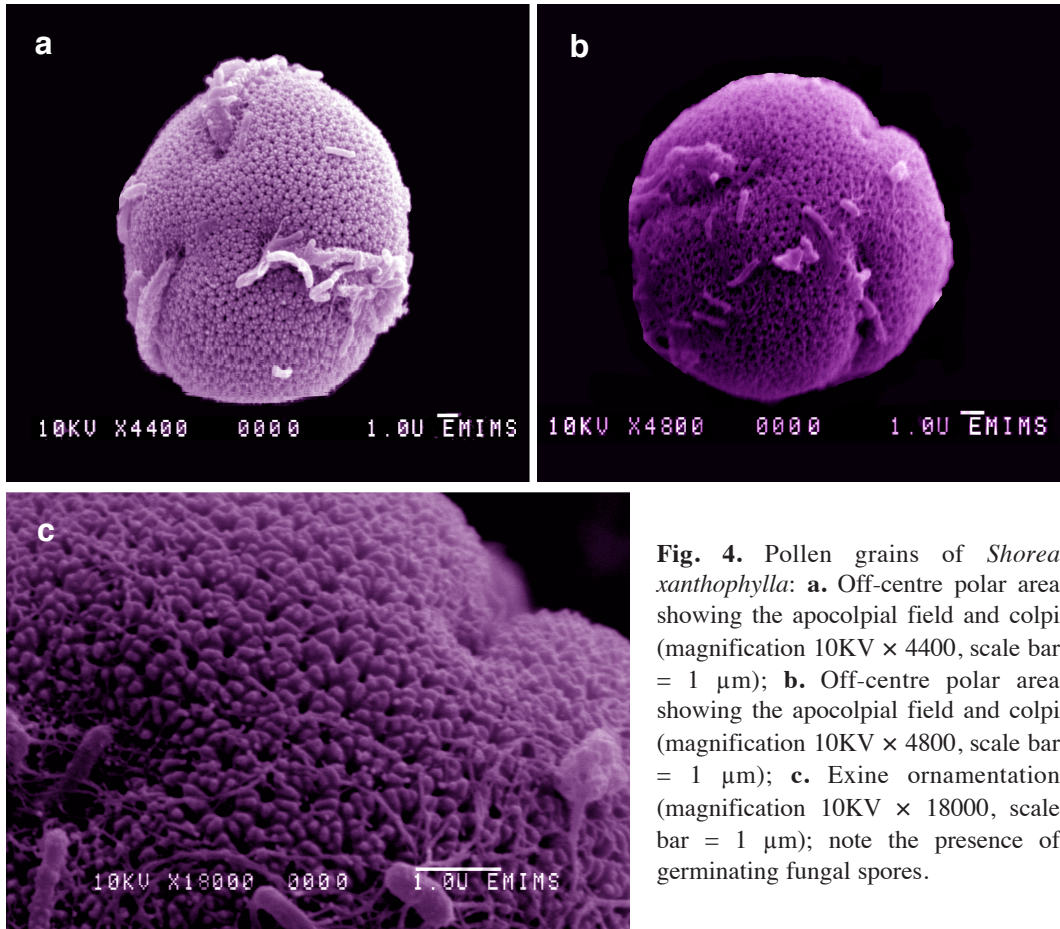


Fig. 4. Pollen grains of *Shorea xanthophylla*: **a.** Off-centre polar area showing the apocolpial field and colpi (magnification 10KV \times 4400, scale bar = 1 μm); **b.** Off-centre polar area showing the apocolpial field and colpi (magnification 10KV \times 4800, scale bar = 1 μm); **c.** Exine ornamentation (magnification 10KV \times 18000, scale bar = 1 μm); note the presence of germinating fungal spores.

Shorea leprosula

The pollen grains were medium in size ($P/E: 24.0 \pm 0.4 \times 21.5 \pm 0.4 \mu\text{m}$, $n = 50$) and subspheroidal, prolate spheroidal and subprolate ($P/E = 1.12 \pm 0.02$). They had a lobate, triangular outline in polar view with a curved distal face (Fig. 5a). They were subspheroidal to rectangular in equatorial view (Fig. 5b). The pollen grains were tricolpate, with two obvious wide colpi (CW: $6.0 \pm 0.8 \mu\text{m}$, $n = 6$ colpi on three pollen grains) and a probable short shallow colpus on the curved distal face (observed with LM). The colpi were relatively long (CL: 8.1 ± 0.54 , $n = 6$ colpi on three pollen grains). Exine ornamentation was microreticulate with croton patterning. The muri were undulating and narrow (M: $0.35 \pm 0.02 \mu\text{m}$, $n = 10$ on one pollen grain) and scored into triangular, irregular and spherical segments (Fig. 5c). Lumina were broad (L: $0.56 \pm 0.04 \mu\text{m}$, $n = 10$ on one pollen grain) elongate, spherical or elliptical.

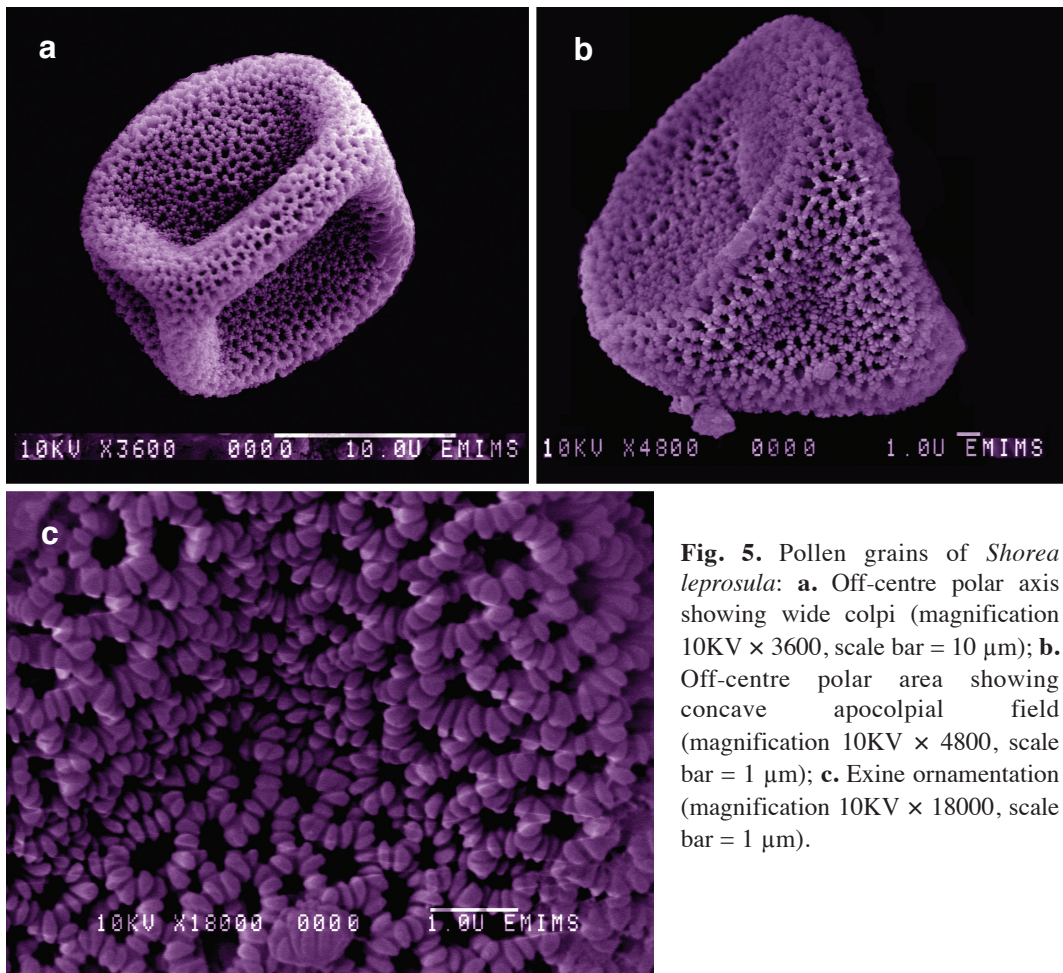


Fig. 5. Pollen grains of *Shorea leprosula*: **a.** Off-centre polar axis showing wide colpi (magnification $10\text{KV} \times 3600$, scale bar = $10 \mu\text{m}$); **b.** Off-centre polar area showing concave apocolpial field (magnification $10\text{KV} \times 4800$, scale bar = $1 \mu\text{m}$); **c.** Exine ornamentation (magnification $10\text{KV} \times 18000$, scale bar = $1 \mu\text{m}$).

The size and morphology of Dipterocarpaceae pollen

The basic tricolpate structure of Dipterocarpoideae pollen observed in this study concurs with previous descriptions and the images presented by Maury *et al.* (1975) and Talip (2008). Only two of the species we sampled at Sepilok Forest Reserve, *P. tomentella* and *S. leprosula*, have been examined in previous studies. Our description of *P. tomentella* is similar to that of Talip (2008), with the exception that she described values of mean polar length (P) and equatorial diameter (E) that were substantially greater than those we found: 55.75 μm (P) and 40.75 μm (E), respectively, compared to values of 26 μm (P) and 24 μm (E), respectively, for our samples. Talip's (2008) larger P and E measurements also lead to a larger P/E ratio, which implies a different shape class (prolate) whereas we found that *P. tomentella* pollen grains were mostly spheroidal. This disparity might be caused by inherent intraspecific variation in pollen size and shape, or it could have arisen because of different methodologies: Talip (2008) used pollen from herbarium specimens collected in 1960, whereas our study is based on fresh material. In addition, the use of acetolysis for preparation of pollen samples prior to examination by Talip (2008) may have increased pollen size relative to our pollen samples that were not acetolysed (Hesse & Waha, 1989). Alternatively size differences could be an artifact arising from measurement error: Talip's (2008) image (Fig. 1g) of *P. tomentella* displays a 10 μm scale bar from which we estimate that equatorial diameter (E) should have been reported as about 23 μm and not the value of 40.75 μm actually quoted by Talip (2008). This re-assessment makes the values of equatorial diameter of *P. tomentella* pollen almost identical in Talip's (2008) study (23 μm) and ours (24 μm).

Ultimately, the cause of this discrepancy between the SEM images and the mean dimensions reported by Talip (2008) remains unknown, and further research is required to resolve the issue.

We found no previous description of the pollen of *S. multiflora* or *S. xanthophylla*. The basic outline, tricolpate structure, and microreticulate crotonoid patterning were similar for the two species. There was a significant difference in polar axis length between them (Kruskal-Wallis $\chi^2 = 13.9$, $df = 1$, $p < 0.001$), but no significant difference in equatorial diameter (Kruskal-Wallis $\chi^2 = 0.822$, $df = 1$, $p = 0.365$). The exine ornamentation was microreticulate crotonoid in both species. Similarly, the average size of the muri and lumina differed by only 0.01 μm and 0.02 μm , respectively. These similarities were anticipated because these species share similar floral and anther characteristics and belong to the same section *Richetioides* of *Shorea* and are therefore closely related.

We found only one published description of *S. leprosula* (Maury *et al.*, 1975), which detailed the shape as spherical and tricolpate, and with a circular equatorial outline. By contrast, we found pollen grains of *S. leprosula* to be subspheroidal in equatorial view and lobate triangular in polar view (Fig. 5). Maury *et al.* (1975) do not present an SEM image of *S. leprosula* pollen, but their LM images support their description and contrast with our

SEM images. In principle, the lobed shape of *S. leprosula* pollen grains in the SEM images of our study might have resulted from dehydration or collapse during sample preparation (Price & Ayers, 2008). Although we observed a similar lobed shape in images derived from LM, mature pollen grains change shape and volume after removal from the anther (Payne, 1972; Hesse, 2000) and these changes may account for the divergence in pollen morphology of *S. leprosula* between our study and Maury *et al.* (1975). Notwithstanding this caveat, it remains a possibility that pollen grains of *S. leprosula* display intraspecific variation in shape that would merit further study.

There are perceptible differences between the pollen morphology of *Parashorea tomentella* and the three *Shorea* species. There are also notable differences in outline and shape class between *S. leprosula* of section *Mutica* and the two species (*S. multiflora* & *S. xanthophylla*) of section *Richetioides*. However, in the absence of a broader survey of pollen morphology among the sections of *Shorea* and the genera of the Dipterocarpaceae, it is not possible to infer whether pollen identification based on shape and outline would be possible. Conversely, the pattern and dimensions of exine ornamentation were distinct among the four species in this study, except in the case of the two species of *Shorea* section *Richetioides*, and these traits might be used for distinguishing among these taxa. A broader review of dipterocarp pollen morphology (Maury *et al.*, 1975) found that pollen from related dipterocarp taxa shared similarities in exine ornamentation. We conclude that, despite the consistencies in pollen morphology amongst closely related species, pollen shape, size and exine ornamentation do not differ consistently among the genera of dipterocarps or the sections within the large genus *Shorea*. However, the association between the similarities in pollen morphology and relatedness suggests that pollen morphology may be phylogenetically constrained in the Dipterocarpaceae (Talip, 2008).

Pollen volume was relatively invariable within a species (CVs 9.8–12.2 %), but mean polar axis length differed among the species (Kruskal-Wallis $\chi^2 = 156$, $df = 3$, $P = < 0.001$). This difference in pollen volume was positively correlated to flower size ($R^2 = 0.92$, $P = 0.029$; Fig. 1). Similarly, although the relationship between pollen volume and flower size was not significant for the species examined by Talip (2008), the trend was positive but offset above the fitted line for the species we studied (Fig. 1). Müller (1979) also found a correspondence between pollen dimensions and flower size for the Dipterocarpaceae. A relationship between flower size and pollen size may be associated with a positive correlation between flower size and stigma depth and/or style length (Cruden, 2009; Ortega Olivencia *et al.*, 1997), although further work is required to examine these relationships within the Dipterocarpaceae.

CONCLUSIONS

The four species we examined could be grouped on the basis of exine ornamentation, which would allow us to distinguish between them except in the case of the two closely-related

species *S. multiflora* and *S. xanthophylla*. Our descriptions of the pollen size of *P. tomentella* and the shape and morphology of *S. leprosula* differ from previously published accounts, and we recommend that further research should be conducted to determine the cause of these differences. Finally, we found that pollen volume was positively correlated with flower size, across the four species we sampled, which corresponds to relationships inferred from larger data-sets. This relationship may have functional significance for dipterocarp reproductive biology and would be a worthy topic of future research.

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