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ORIGINAL ARTICLE

Pollen morphology and ultrastructure of several *Gnetum* species: an electron microscopic study

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Abstract Pollen grains of five *Gnetum* species have been studied in scanning and transmission electron microscopy: *G. africanum*, *G. funiculare*, *G. indicum*, *G. leptostachyum*, and *G. macrostachyum*. The exine ultrastructure was described for the first time for *G. funiculare*, *G. leptostachyum*, and *G. macrostachyum*. The pollen grains are small, inaperturate, and microechinate. The sporoderm includes a rather thin tectum, granular infratectum, and lamellate endexine. The foot layer is indistinct or absent in *G. africanum*, *G. funiculare*, and *G. macrostachyum* and thin in *G. indicum* and *G. leptostachyum*. *Gnetum africanum* differs from other studied species of the genus in having smaller supratectal microechini. They occur on considerably raised exine regions (islands) that are interpreted as an equivalent to the plicae in *Ephedra* and *Welwitschia*. In Asian species of *Gnetum*, a microechinus and area around it are interpreted as equivalent to the islands of *G. africanum* and the plicae in *Ephedra* and *Welwitschia*. The infratectum in *G. africanum* consists of few, widely spaced large granules in contrast to small densely packed granules of other studied *Gnetum* species. A comparison of the published and original data on extant pollen of Gnetales and fossil ephedroid pollen shows a great similarity in the sporoderm ultrastructure. Absence of *Gnetum*-like pollen in the fossil record may be due to their thin ectexine, possible separation of the ect- and endexine or misinterpretation.

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Keywords Gnetales · *Gnetum* pollen · Granular ultrastructure · Microechinate sculpture

Introduction

Pollen grains of *Ephedra*, *Welwitschia* and *Gnetum* have been studied by means of light and electron microscopy (see review in Osborn 2000; Tekleva and Krassilov 2009). Despite opinions on the absence of any connection between pollen of *Gnetum* and the other two genera of Gnetales in early works (e.g., Wodehouse 1935; Gullväg 1966), it has become evident that the three genera share the same pattern of the sporoderm ultrastructure (e.g., see Osborn 2000). The only exception is *G. africanum*, the only studied African species of this genus, which was reported to have a baculate infratectum in contrast to all other studied members of Gnetales (Orel et al. 1986).

The microechinate sculpture and spheroidal shape of *Gnetum* pollen are distinctly different from the polyplicate ellipsoidal pollen of *Ephedra* and *Welwitschia*. What could have led to such a distinction and how it was acquired? Why are pollen grains of *Gnetum* type almost absent in the fossil record? These and more questions are still pending when we think about this enigmatic group of seed plants, and accumulation of further data is needed to resolve at least some of them.

To answer these questions we describe and discuss pollen morphology and ultrastructure of five *Gnetum* species. These species had been previously studied by the author but their description was not published and the data (several photos) were only used for a comparison with fossil gnetophytes in Tekleva et al. (2006) and Tekleva and Krassilov (2009). The sporoderm ultrastructure of *G. funiculare*, *G. leptostachyum*, and *G. macrostachyum* is

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described for the first time. Additionally, our data on the pollen wall of modern *Welwitschia* and *Ephedra*, and fossil ephedroid pollen are used for comparison.

Materials and methods

Extant pollen grains were obtained from the palynological collection of the Department of Higher Plants, M.V. Lomonosov Moscow State University (*Welwitschia mirabilis* Hook.f., *Ephedra monosperma* J.G.Gmel. ex C.A.Mey., and *Gnetum indicum* Merr.), from the herbaria of the Botanical Institute, St-Petersburg (*Gnetum africanum* Welw. and *G. funiculare* Wight), and from the Biology Department's Herbarium of the Chang-Mai University, Thailand (*G. leptostachyum* Blume and *G. macrostachyum* Hook.f.). For SEM, untreated pollen grains were mounted on SEM stubs (covered with nail varnish) and sputter-coated with gold–palladium (Camscan, Hitachi) or gold (Tescan). The pollen grains were observed and photographed under SEMs: Camscan (pollen of *Welwitschia*, *Ephedra*, and all *Gnetum* species, except for *G. indicum*), Hitachi (fossil ephedroid pollen) with accelerating voltage 20 kV, and Tescan (*G. indicum*) with accelerating voltage 30 kV. For TEM, individual pollen and fragments of sporangia (hydrated) were fixed with 1 % OsO_4, dehydrated in an ethanol series, stained with uranyl acetate, dehydrated in acetone, and embedded in epoxy resin according to Meyer-Melikian et al. (2004). At least 10 pollen grains were studied under TEM for each species and 20 pollen grains under SEM for each species. Fossil pollen grains come from the Furao Formation, borehole XHY2008, late Maastrichtian, Amur (Heilongjiang) River area, Zeya-Bureya Basin (see Markevich et al. 2011 for detailed information). They were picked from the residue and studied with light (LM), scanning (SEM) and transmission (TEM) electron microscopy. The pollen grains were sectioned with LKB-3 (pollen of *Welwitschia*, *Ephedra*, and all *Gnetum* species, except for *G. indicum*) and Leica UC6 (*G. indicum*, fossil ephedroid pollen) ultramicrotomes. The ultrathin sections were post-stained with lead citrate and examined under Jeol 100 B (all studied pollen grains) and Jeol 400 (fossil ephedroid pollen) TEMs with accelerating voltage 80 kV.

In this paper slightly or considerably raised exine areas with microechini in *Gnetum* pollen are called islands (Fig. 1b–e, g–i, arrowheads) and areas between them—valleys. These are not palynological terms but they make it easy to describe the sporoderm ultrastructure of *Gnetum* pollen. Otherwise, the pollen terminology follows Hesse et al. (2009).

Results

Gnetum africanum Welw. (Figs. 1a, b, 2a–d)

SEM

The pollen diameter is about 12.2 μm. The sculpture is microechinate. Microechini are very small, about or less than 0.2 μm high, rather widely spaced, blunt, with several (5–20) microechini on considerably raised exine areas, the latter looking like islands (Fig. 1a, b).

TEM

The exine thickness is about 1.3 μm in island regions (Fig. 2a, b) and about 0.45 μm between them (Fig. 2a, c). In islands, the ectexine consists of a homogeneous imperforate tectum and granular infratectum (Fig. 2d). The tectum thickness is more or less uniform within the island, about 0.29 μm, except for the central (top) part where the tectum has a conical process (a microechinus) and may reach up to 0.46 μm thick (Fig. 2b). The tectum decreases in thickness towards the valley regions (Fig. 2b, c). The infratectal granules are large, 0.2–0.6 μm in diameter, and widely spaced; one to four granules were observed per island in each particular section (Fig. 2a, b, d). The foot layer is indistinct or absent. The endexine is less electron dense than the ectexine, about 0.45 μm thick, uniform in thickness around the pollen grain. It consists of five to eight anastomosing lamellae, which are clearly distinct in the outer part and closely appressed to each other in the inner part (Fig. 2a–d). The valley is represented by the endexine and probably by a thin tectum (Fig. 2c).

Gnetum funiculare Wight (Figs. 1c, d, 2e–g)

SEM

The pollen diameter is about 14 μm. The sculpture is microechinate. Microechini are about 0.5 μm high, regularly distributed, blunt, are located on small, distinct and considerably raised exine areas (islands); there are one or two microechini per each island (Fig. 1c, d).

TEM

The exine thickness is about 1.3 μm in island regions and about 0.8 μm between them (Fig. 2e). In islands, the ectexine consists of a homogeneous imperforate tectum and granular infratectum (Fig. 2f, g). The tectum thickness is more or less uniform within the island, about 0.09 μm, it thins towards the margins (towards the valley, Fig. 2g). The

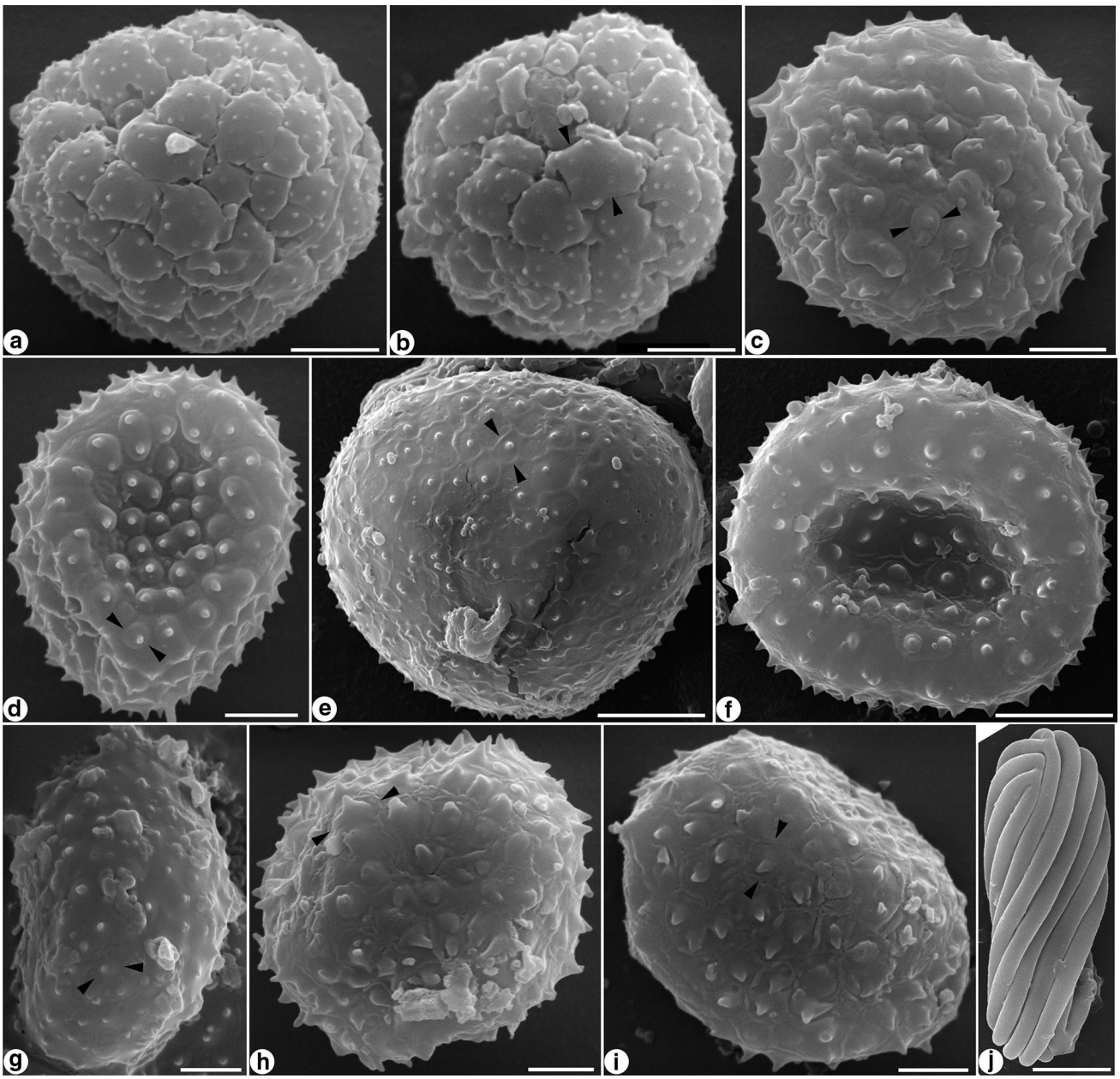


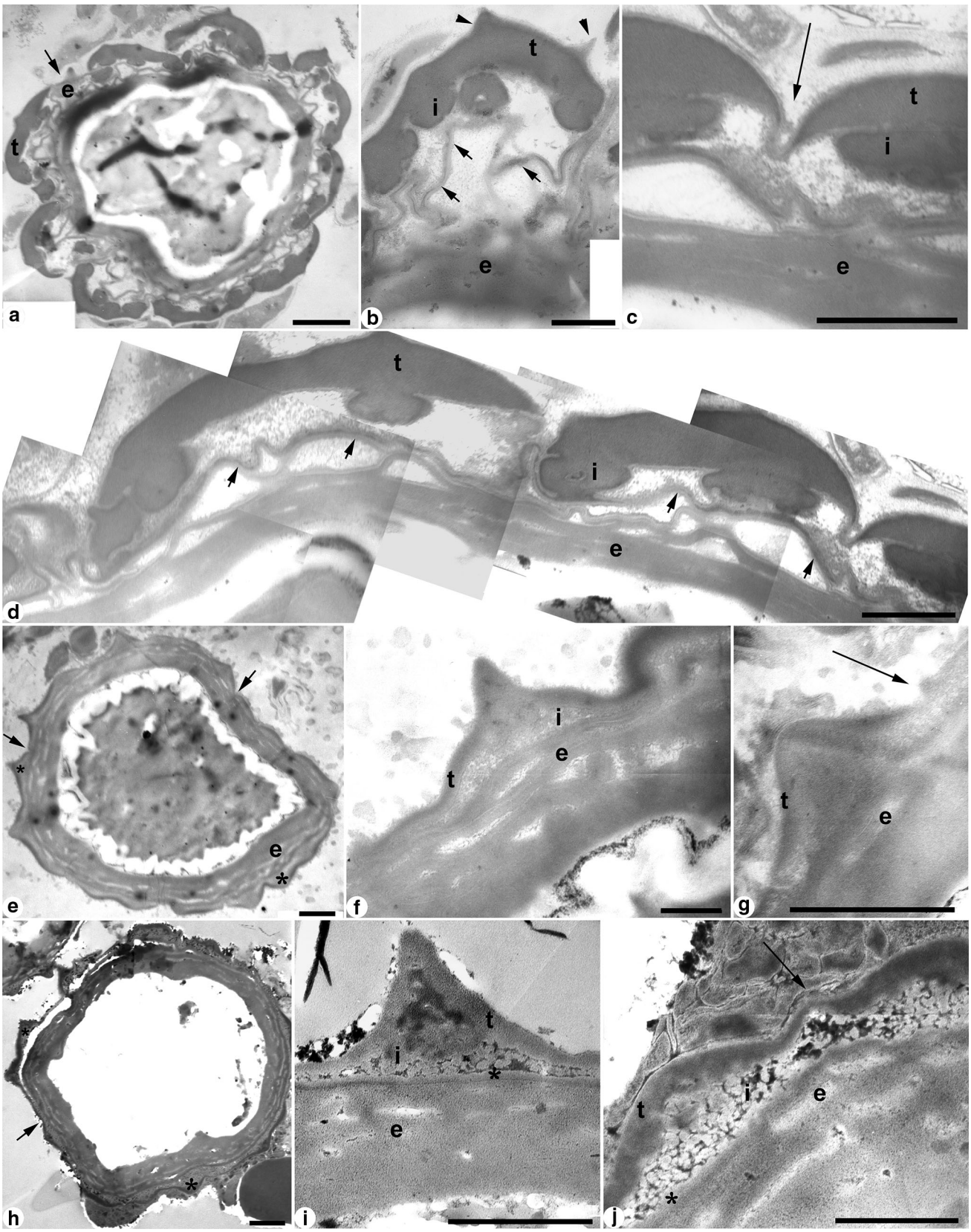
Fig. 1 Extant *Gnetum* pollen and fossil ephedroid pollen, SEM. **a**, **b** *G. africanum*. **c**, **d** *G. funiculare*. **e**, **f** *G. indicum*. **g** *G. leptostachyum*. **h**, **i** *G. macrostachyum*. **j** Fossil ephedroid pollen from Furao Formation, Late Maastrichtian. *Arrowheads* border island regions. *Scale bar* 3 μm for **a**–**d**, **g**–**i**; 5 μm for **e**, **f**; 10 μm for **j**

infratectal granules are small, densely packed, scarcely discernable, about 0.02 μm in diameter. The foot layer is indistinct or absent. The endexine is about the same electron density as the ectexine. It is uniform in thickness around the pollen grain, about 0.8 μm, and consists of six to eight anastomosing lamellae, which are clearly distinct in the outer part and densely appressed to each other in the inner part of this layer (Fig. 2e, f). The valley is represented by the endexine and a thin tectum (Fig. 2f, g).

Gnetum indicum Merr. (Figs. 1e, f, 2h–j)

SEM

The pollen diameter is 18 μm. The sculpture is microechinate. Microechini are about 0.5 μm high, regularly distributed, blunt, there are one or rarely two echini per each slightly raised area (island, Fig. 1e, f).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◀**Fig. 2** Sporoderm structure of *Gnetum africanum* (**a**–**d**), *G. funiculare* (**e**–**g**), and *G. indicum* (**h**–**j**), TEM. **a** Section through a whole pollen grain, *arrow* indicates one of the valley regions. **b** Island region, *arrowheads* point to the microechini, *arrows* indicate endexine lamellae. **c** Valley region (*arrow*). **d** Part of the sporoderm, *arrows* indicate endexine lamellae. **e** Section through a whole pollen grain, *arrow* indicates one of the valley regions, *asterisk* indicates one of the island regions. **f** Island region. **g** Part of the sporoderm, section through the island region without microechinus, *arrow* indicates valley region. **h** Section through a whole pollen grain, *arrow* indicates one of the valley regions, *asterisk* indicates one of the island regions. **i** Island region, *asterisk* indicates probable foot layer. **j** Part of the sporoderm, section through the island region without microechinus, *arrow* indicates valley region, *asterisk* indicates probable foot layer. *t* tectum, *i* infratectum, *e* endexine. *Scale bar* 1.25 µm for **a**; 0.5 µm for **b**–**d**, **i**, **j**; 1 µm for **e**, **g**, **h**; 0.4 µm for **f**. **f** Reproduced from the same original image as Fig. 6 in the Plate 4 in Tekleva et al. (2006)

TEM

The exine thickness is about 1.1 µm in island regions (Fig. 2h, i) and about 0.6 µm in valleys (Fig. 2h, j). In islands, the ectexine consists of a homogeneous imperforate tectum, granular infratectum, and thin foot layer (Fig. 2i). The tectum thickness is more or less uniform within the island, about 0.04 µm; it thins towards the margins. The infratectal granules are small, densely packed, about 0.05 µm in diameter. The granules that lie on the endexine are often smaller than the granules located closer to the tectum. The foot layer is about 0.02 µm thick. The endexine is slightly less electron dense than the ectexine, about 0.6 µm thick, uniform in thickness around the pollen grain. It consists of seven to eight anastomosing lamellae, which are clearly distinct in the outer part and closely appressed to each other in the inner part (Fig. 2h–j). The valley is represented by the endexine, foot layer and thin tectum (Fig. 2j).

Gnetum leptostachyum Blume (Figs. 1g, 3a–c)

SEM

The pollen diameter is about 12.3 µm. The sculpture is microechinate. Microechini are about 0.4 µm high, regularly distributed, blunt. Islands are indistinct and only slightly raised, each bears one or two echini (Fig. 1g).

TEM

The exine thickness is about 1.0 µm in island regions (Fig. 3a, b) and about 0.52 µm between them (Fig. 3a, c). In islands, the ectexine consists of a homogeneous imperforate tectum, granular infratectum, and thin foot layer (Fig. 3b). The tectum thickness is more or less uniform within the island, about 0.05 µm, it thins towards the margins. The infratectal granules are small, densely packed, about 0.04 µm in diameter. The granules that lie on the endexine are often smaller than the granules located closer to the tectum. The foot layer is less than 0.02 µm thick. The endexine is about the same electron density as the ectexine, uniform in thickness around the pollen grain and about 0.51 µm. It consists of six to ten anastomosing lamellae, which are clearly distinct in the outer part and closely appressed to each other in the inner part (Fig. 3a–c). The valley is represented by the endexine, foot layer and thin tectum (Fig. 3c).

Gnetum macrostachyum Hook.f. (Figs. 1h, i, 3d–f)

SEM

The pollen diameter is about 14.4 µm. The sculpture is microechinate. Microechini are about 0.6–0.7 µm, sometimes almost up to 1 µm. They are regularly distributed, blunt and with slightly curved tips. There is one echinus per each distinct, weakly raised island (Fig. 1h, i).

TEM

The exine thickness is about 1.3 µm in island regions and about 0.6 µm between them. In islands, the ectexine consists of a homogeneous imperforate tectum and granular infratectum (Fig. 3e). The tectum thickness is more or less uniform within the island, about 0.12 µm, it thins towards the margins. The infratectal granules are small, densely packed, about 0.04 µm in diameter. The granules that lie on the endexine are often smaller than the granules located closer to the tectum. The foot layer is indistinct or absent. The endexine is about the same electron density as the ectexine, uniform in thickness around the pollen grain, about 0.58 µm thick. It consists of six to ten anastomosing lamellae, which are clearly distinct in the outer part and closely appressed to each other in the inner part (Fig. 3d–f). The valley is represented by the endexine and thin tectum (Fig. 3f).

Discussion

Pollen morphology of *Gnetum* species

Gnetum comprises about 40 species in Africa, South America, and Southeast Asia (Biye et al. 2014; Hou et al. 2015). Pollen grains are small (11–20 µm in diameter), spheroidal, and microechinate. For some species (e.g., *G. africanum* in Orel et al. 1986) the sculpture is described as "tuberculate" with pointed microechini on the tuberculum surface. Pollen grains of *Gnetum* are usually considered inaperturate, although a leptoma or pore-like area is

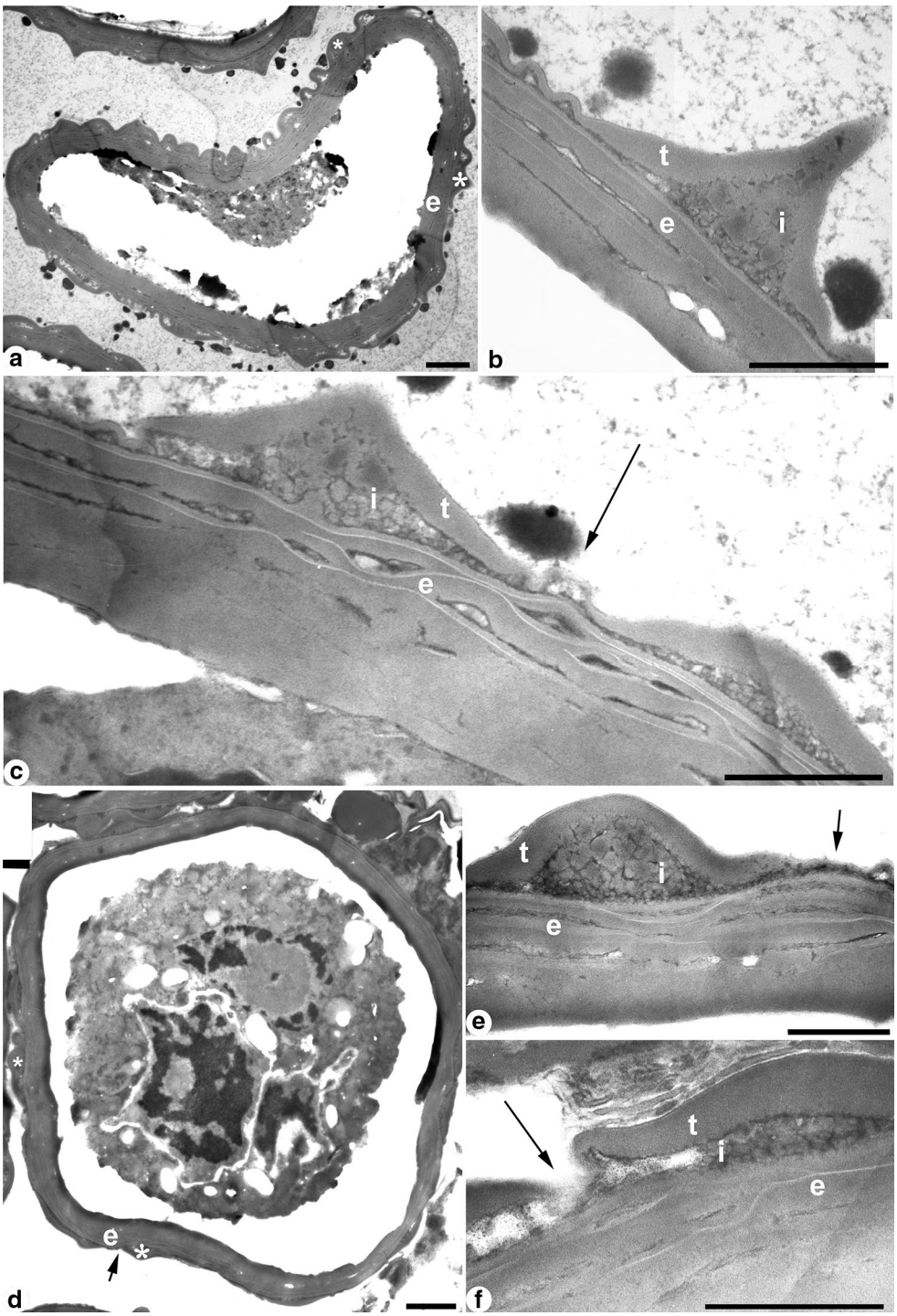


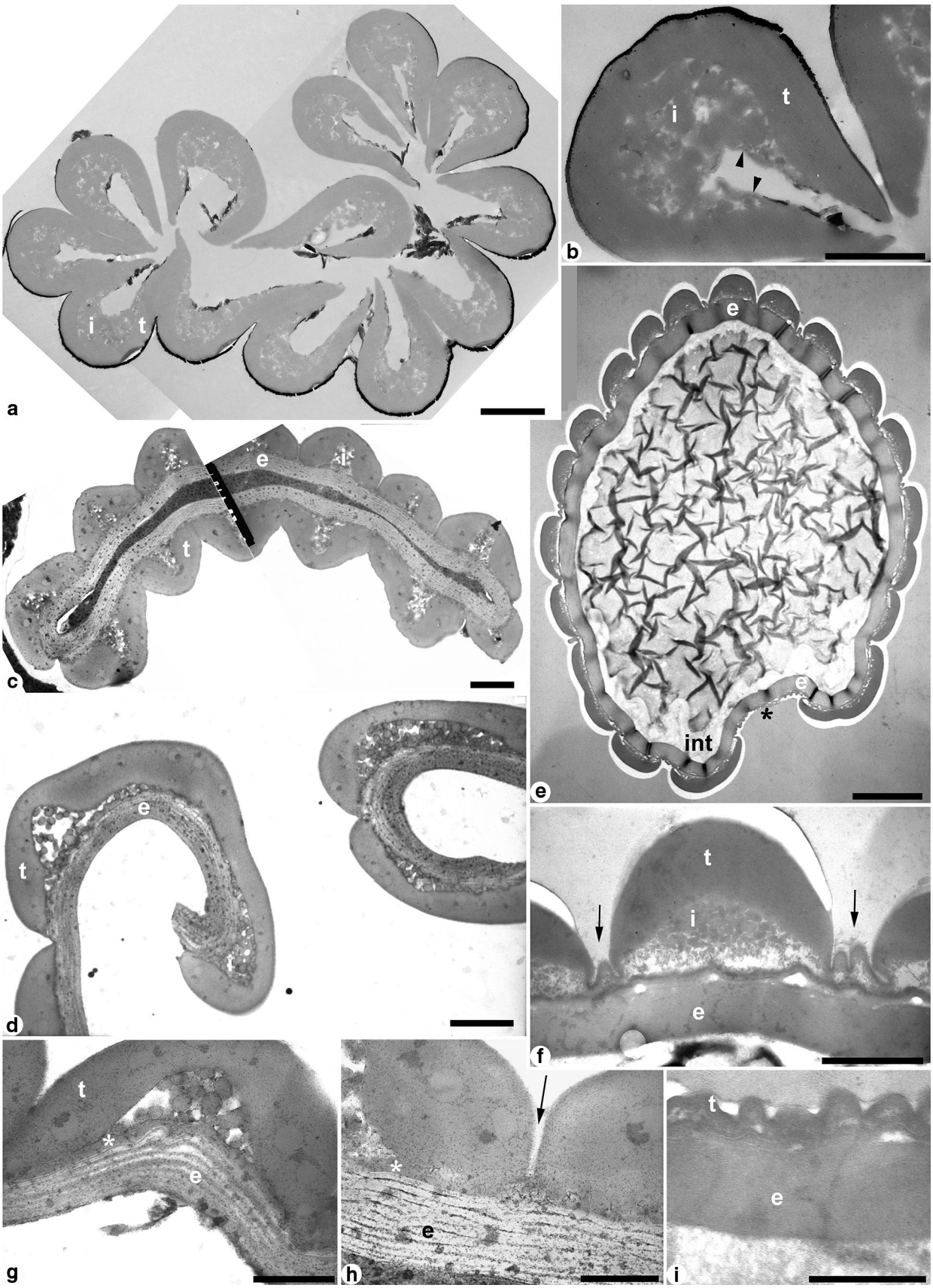
Fig. 3 Sporoderm structure of *Gnetum leptostachyum* (**a**–**c**), *G. macrostachyum* (**d**–**f**), TEM: **a** Section through a whole pollen grain; **b** island region; **c** part of the sporoderm; **d** section through a whole pollen grain; **e** island region; **f** part of the sporoderm. *t* tectum, *i* infratectum, *e* endexine. *Arrows* indicate valley regions; *asterisk* indicates an island. *Scale bar* 1 μm for **a**, **d**; 0.5 μm for **b**, **c**, **e**, **f**. **b** Reproduced from the same original image as Fig. 4 in the Plate 4 in Tekleva et al. (2006)

mentioned in some studies (Erdtman 1965; Kuprianova 1983; Orel et al. 1986). The presence of an aperture was not confirmed by other authors and in the present study no aperture area was observed in SEM or TEM.

In the pollen under study there is a difference between the African (*G. africanum*) and other (Asian) species (Table 1). In *G. africanum* microechini are very small (0.2 μm versus 0.4–0.7 μm in other species) and there are

Table 1 Pollen features of studied *Gnetum*, *Ephedra*, and *Welwitschia* species

Taxon	*Gnetum africanum* Welw.	*Gnetum funiculare* Wight	*Gnetum indicum* Merr.	*Gnetum leptostachyum* Blume	*Gnetum macrostachyum* Hook.f.	*Ephedra monosperma* J.G.Gmel. ex C.A.Mey.	*Welwitschia mirabilis* Hook.f.
Average pollen size, μm	12.2	14	18	12.3	14.4	37 (long axis)	51 (long axis)
Aperture condition	Inaperturate	Inaperturate	Inaperturate	Inaperturate	Inaperturate	Inaperturate	Monosulcate
Sculpture	Microechinate; microechini are about or less than 0.2 μm high; there are several (5–20) microechini per each island	Microechinate; microechini are about 0.5 μm high; there are one or two microechini per each island	Microechinate; microechini are about 0.5 μm high; there are one or two microechini per each island	Microechinate; microechini are about 0.4 μm high; there are one or two microechini per each island	Microechinate; microechini are about 0.6–0.7 μm, sometimes almost up to 1 μm high; there is one microechinus per each island	Polyplicate, with psilate plicae	Polyplicate, with psilate plicae
Average exine thickness, μm	1.3 μm in island area, 0.45 μm in valley region	1.3 μm in island area, 0.8 μm in valley region	1.1 μm in island area, 0.6 μm in valley region	1.0 μm in island area, 0.52 μm in valley region	1.3 μm in island area, 0.6 μm in valley region	Up to 2.2 μm in plica region and about 0.44 μm in valley region	Up to 1.2 μm in plica region and about 0.2 μm in valley region
Tectum	More or less uniform in thickness, about 0.29 μm, except for the central (top) part where it is up to 0.46 μm	More or less uniform in thickness, about 0.09 μm	More or less uniform in thickness, about 0.04 μm	More or less uniform in thickness, about 0.05 μm	More or less uniform in thickness, about 0.12 μm	About 0.7 μm, but it is thinner at the crest (about 0.3 μm)	More or less uniform in thickness, about 0.57 μm
Infratectum	Of large granules 0.2–0.6 μm in diameter, one to four granules per each island	Of small numerous granules about 0.02 μm in diameter	Of small numerous granules about 0.05 μm in diameter, with gradient in granule size	Of small numerous granules about 0.04 μm in diameter, with gradient in granule size	Of small numerous granules about 0.04 μm in diameter, with gradient in granule size	Of small numerous granules about 0.12 μm in diameter	Of small numerous granules about 0.04 μm in diameter
Foot layer	Indistinct or absent	Indistinct or absent	0.02 μm thick	Less than 0.02 μm thick	Indistinct or absent	0.07 μm thick	0.04 μm thick
Exine structure in valley region	Endexine and, probably thin tectum	Endexine and thin tectum	Endexine, foot layer, and thin tectum	Endexine, foot layer, and thin tectum	Endexine and thin tectum	Endexine and thin ectexine (?foot layer)	Endexine and thin tectum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◀**Fig. 4** Sporoderm structure of fossil ephedroid pollen (**a**, **b**), and extant pollen of *Ephedra monosperma* (**c**, **d**, **g**, **h**), and *Welwitschia mirabilis* (**e**, **f**, **i**). **a** Section through a whole pollen grain. **b** Plica region, *arrowheads* indicate remnants of the inner layer probably representing foot layer or endexine; **c**. section through a whole pollen grain; **d** part of the sporoderm of the shed exine; **e** section through a whole pollen grain, *asterisk* indicates sulcus region; **f** part of the sporoderm, plica and valley (*arrow*) regions; **g** plica region; **h** valley region (*arrow*); **i** enlarged image of apertural exine. *t* tectum, *i* infratectum, *e* endexine, *int* intine. *Scale bar* 1.25 μm for **a**, **c**; 1 μm for **b**, **d**, **f**; 2.5 μm for **e**; 0.5 μm for **g**–**i**

more than two of them per each raised area (island). One or two small or rather high (in case of *G. macrostachyum*) microechini per each island are observed in pollen of the other studied species.

Erdtman (1954, 1965) and Kuprianova (1983) recognized three principal pollen types in *Gnetum* according to the pollen morphology observed in transmitted light. They coincide with African, neotropical, and Asian species. The pollen type of two African species was described as "Sexine insulous or nearly so. Insulae tectate; tegilla supported by baculum-like elements"; neotropical species were characterized by pollen with a "pilate or probably pilate sexine"; Asian species were characterized by "spinulose" pollen (Erdtman 1965). The present study showed that in African species (or, at least, in *Gnetum africanum*) there are no "baculum-like elements" or "pilate sexine", and the infractectum is characterized by large granules.

Later, Gillespie and Nowicke (1994) argued that there were two rather than three pollen types distinguished in LM and SEM: the first comprises Asian species and the second includes African and neotropical species. The types mainly differ by a continuous or discontinuous tectum and (micro)echinus size ("spines" or "spinules" in terminology used by Gillespie and Nowicke). The first type is characterized by "an imperforate, uniform tectum with conical, blunt spines" and the second type shows "an irregularly thickened and discontinuous tectum with spines". In LM the exine "appears of uniform thickness with clearly visible spines" in pollen of the first type, and it is "irregularly thickened with barely visible spines" in pollen of the second type. In SEM the spinules are "distinctly smaller, more rounded and more numerous" in pollen of the second type. Gillespie and Nowicke (1994) noted that some neotropical species like *G. schwackeanum* Taub. ex A.Schenk have exine sculpturing intermediate between *G. africanum* and typical neotropical *Gnetum* species like *G. urens* (Aubl.) Blume. Indeed, pollen of the neotropical *Gnetum* species studied (in SEM) show either small microechini like those in *G. africanum* and/or are characterized by more than two microechini per each island. For example, *G. nodiflorum* Brongn. (Osborn 2000, Figure 16) looks quite similar to *G. africanum*. Unfortunately, the sporoderm ultrastructure has not been studied for any neotropical *Gnetum* species and the exine structure of only one African *Gnetum* species has been studied. Considering the striking difference of the infratectum of *G. africanum* it is necessary to study the pollen ultrastructure of neotropical and other African species of *Gnetum* to confirm either of the two ideas (Erdtman's or Gillespie and Nowicke's) on the differentiation of *Gnetum* pollen into two or three principal types. If a similar infratectum of large granules is found in pollen of neotropical *Gnetum* species and other African species, then two principal pollen types as distinguished by Gillespie and Nowicke (1994) will be confirmed.

Molecular phylogenetic studies (e.g., Hou et al. 2015; Won and Renner 2003, 2005) support the monophyly of South American, African, and Asian groups of *Gnetum* species with the South American (neotropical) clade being basal, and the African clade sister to the Asian clade. There are some differences in the results of molecular studies and future studies are needed. However, the present study shows that these three clades are also well distinguished by palynological characters and they can help in the further classification of *Gnetum*.

Infratectum structure of *Gnetum* species

The sporoderm ultrastructure has been studied for *Gnetum africanum* (Orel et al. 1986; our data), *G. cleistostachium* C.Y.Cheng (Yao et al. 2004), *G. gnemon* L. (Gullväg 1966; Hesse 1980; Kurmann 1992), *G. hainanense* C.Y.Cheng (Yao et al. 2004), *G. indicum* (Bernard and Meyer 1972; Meyer-Melikian 1994; our data), *G. funiculare*, *G. leptostachyum* Blume (our data), *G. luofuense* C.Y.Cheng (Yao et al. 2004), *G. macrostachyum* Hook.f. (our data), *G. montanum* Markgr. (Gullväg 1966; Meyer-Melikian 1994; Yao et al. 2004), *G. parvifolium* (Warb.) Cheng, *G. pendulum* (Yao et al. 2004), *G. ula* Brongn. (Gullväg 1966), and *Gnetum* sp. (Zavada 1984a). A granular infratectum was reported for all species, except *G. africanum*. According to Orel et al. (1986) *G. africanum* has a baculate infratectum. Our study of this species (from the same specimen) showed that the baculi are in fact large granules (Fig. 2a–d; see also Tekleva and Krassilov 2009). Unfortunately, photos of the exine ultrastructure were not published for all species. This impedes a comprehensive comparison.

A comparison of pollen morphology and ultrastructure in *Gnetum* and other Gnetales members

Osborn (2000) pointed out that microechini ("spines and spinules" in his paper) of *Gnetum* pollen are not

supratectal. In contrast to echini of most angiosperms they are formed by the tectum and infratectum. Summarizing our data we conclude that this is true for pollen of all studied *Gnetum* species, except for *G. africanum*. Apparently, for most studied species of *Gnetum*, at the ultrastructural level the microechinus and the area around it (Fig. 1c–e, g–i, arrowheads as seen in SEM, and Figs. 2b, d, f, g, i, 3b, c, e as seen in TEM) are equivalent to a plica region found in *Ephedra* and *Welwitschia* pollen (Fig. 1j as seen in SEM, and Fig. 4a–g as seen in TEM). In *G. africanum* an island area with a number of microechini (Fig. 1b, arrowheads) can be considered equivalent to the plica region of *Ephedra* and *Welwitschia* pollen. The possibility that the hollow microechini or islands of *Gnetum* are homologous with the plicae in *Ephedra* and *Welwitschia* has also been incorporated into phylogenetic analysis (Doyle 1996).

Other ultrastructural details also show a great similarity between pollen grains of the three gnetalean genera (Table 1). The tectum is thin in *Gnetum* and rather thick in *Ephedra* and *Welwitschia*. It is uniform in thickness, tapering towards the margins of the echini in *Gnetum* and the margins of the plicae in *Welwitschia* (Figs. 2c, d, f, g, j, 3b, c, e, f for *Gnetum*; Fig. 4e, f for *Welwitschia*), while in *Ephedra* pollen the tectum is thinner at the crest and thickens downwards but it also tapers at the very ends of the plica (El-Ghazaly and Rowley 1997; Fig. 4c, d, g). There are, however, photos of pollen of *Ephedra foliata* in El-Ghazaly and Rowley (1997, Plate 2, Figures 3–5; Plate 3, Figure 2; Plate 4, Figure 5; Plate 5, Figure 1) where no significant difference in the tectum thickness at the crest and at margins of the plica is observed. These photos were of fresh pollen and the overall ectexine thickness is less on these photos than that on other photos of *Ephedra* exine with the usual tectum difference within the plica. The authors do not mention or discuss this disparity. Probably, it may be due to an oblique section orientation or immaturity of the sectioned pollen, although the latter is less probable since Doores et al. (2007) show that the difference in the tectum thickness within the plica is clear at the very early stage of *Ephedra* pollen development.

Although for mature pollen of *Ephedra foliata* Boiss. ex C.A.Mey. (El-Ghazaly and Rowley 1997) and for microspores at early tetrad stage of *E. americana* Hum. & Bonpl. ex Willd. (Doores et al. 2007) occasional small columellae have been reported, the infratectum is granular, consisting of small granules in *Ephedra*, as well as in *Welwitschia*, and in several *Gnetum* species. The granules are of somewhat different size. Pollen grains of some *Gnetum* species (*G. funiculare*, *G. montanum*, and probably *G. cleistostachyum* and *G. pendulum*) have tiny and almost indistinguishable granules and pollen grains of *G. africanum* are unique among species studied so far in showing few large, widely spaced granules.

The foot layer is indistinct or appears to be absent in pollen of some studied *Gnetum* species and some *Ephedra* species or thin, tightly appressed to the endexine in pollen of *Welwitschia* and other *Ephedra* and *Gnetum* species. In *Gnetum* pollen it is sometimes difficult to decide whether there is a thin foot layer or an endexine lamella. The electron density is often almost identical but the layer outside the first white line might be considered as a foot layer, at least in several species (*G. gnemon*: Meyer-Melikian 1994; Kurmann 1992, Fig. 2f; *G. indicum*: this study, Fig. 2 i, j; *G. leptostachyum*: this study, Fig. 3b, c). The endexine is lamellate and uniform in thickness around the pollen grain.

Pollen grains are typically considered inaperturate in *Gnetum* and *Ephedra* and monosulcate in *Welwitschia* (e.g., our data; Doores et al. 2007; discussion in El-Ghazaly and Rowley 1997; Osborn 2000). The valley region (what is usually called "furrow region" for ephedroid pollen) in pollen of *Ephedra* and *Gnetum*, and the aperture region in pollen of *Welwitschia* are represented by a thin tectum, foot layer (if present) and endexine (Table 1; Figs. 2c, d, f, g, j, 3b, c, e, f, 4d, h, i). The valley region in pollen of *Welwitschia* is formed by a thin tectum, thin granular layer, foot layer and endexine (Fig. 4f).

Fossil ephedroid pollen grains identified as *Equisetosporites* sp. and *Ephedripites* sp. from the Lower Cretaceous of Brazil and Italy, respectively, are plicate and inaperturate. Their sporoderm ultrastructure is characterized by a rather thick tectum, infratectum of small granules, foot layer (for *Ephedripites*) and lamellate endexine (Osborn et al. 1993; Trevisan 1980). The fossil ephedroid pollen grain shown here for comparison has a similar aperture condition, morphology, and ultrastructure; the foot layer and endexine were probably not preserved (Fig. 4a, b). While the pollen morphology is quite similar to that of extant *Ephedra* species, the exine structure lacks the thinning at the crest of the plica region and therefore more resembles the exine ultrastructure of extant *Welwitschia* and previously studied fossil ephedroid pollen of *Equisetosporites* and *Ephedripites* (Osborn et al. 1993; Trevisan 1980).

Pollen ontogeny has been studied for *Welwitschia mirabilis* (Zavada and Gabaraeva 1991), *Gnetum gnemon* (Meyer-Melikian 1994), and *Ephedra americana* (Doores et al. 2007). Plica regions are formed at the very early stage of primexine deposition in *Ephedra* and *Welwitschia* (Doores et al. 2007; Zavada and Gabaraeva 1991) and the echinus regions in *Gnetum* are formed similarly as can be concluded from the description and illustrations given in Meyer-Melikian (1994). The infratectal granules are reported to develop after or at the same time as the

endexine in mid tetrad stage for *Gnetum* (Meyer-Melikian 1994) and *Ephedra* (Doores et al. 2007), which is rather unusual for seed plants. In *Welwitschia* pollen infratectal granules also start developing in mid tetrad stage, and the endexine—in late tetrad stage (Zavada and Gabaraeva 1991). Thus, for all three genera endexine development is reported to start in tetrad stage, which is characteristic for gymnosperms (e.g., Kurmann 1990). In most angiosperms the endexine develops in free microspore stage (Blackmore and Barnes 1990; Zavada 1984b). When germinated, pollen grains of *Ephedra* and *Gnetum* shed the exine completely (El-Ghazaly et al. 1998; Thompson 1916; Abercrombie et al. 2011), while in *Welwitschia* pollen it remains as a cap (Rydin and Friis 2005). We can see that in this group (Gnetales), the sporoderm ultrastructure plays a key role confirming the relationship between its members.

A possible interrelation of the exine structure in *Gnetum* species and their pollination mode, harmomegathy and preservation potential

Obviously, pollen grains of the three genera of the Gnetales show a similar type of the exine structure, but why have *Gnetum* pollen grains developed such a distinct microechinate exine sculpture? Several aspects can be considered. Different pollination syndromes can cause diversity in pollen morphology and ultrastructure. Pollination of several species of the three genera has been studied (see review in Endress 1996; Kato et al. 1995; Niklas 2015 and references therein; Wetschnig and Depish 1999). Entomophily was shown for *Welwitschia* by Wetschnig and Depish (1999) though they did not exclude some insignificant role of wind pollination as well. Some species of *Ephedra* are thought to be anemophilous while others are considered exclusively or partly entomophilous (e.g., Bolinder et al. 2015; Buchmann et al. 1989; Meeuse et al. 1990; Niklas 2015). The studied *Gnetum* species were also shown to be entomophilous (Kato et al. 1995 and references therein). Pollen grains of the three genera are reported to be sticky, though they lack pollenkitt (Hesse 1980, 1984; Kato et al. 1995; Meeuse et al. 1990; Wetschnig and Depish 1999).

Bolinder et al. (2015) have revealed a certain correlation between differences in settling velocity and the density of infratectal granules and tectum thickness in entomophilous and anemophilous species of *Ephedra*. As mentioned above, the sporoderm morphology and ultrastructure of *Gnetum* pollen differ from those of *Ephedra* in the sculpture, tectum thickness and (sometimes) granule size. The spheroidal shape and microechinate exine sculpturing might represent a different way of adaptation to entomophily in *Gnetum* lineage. The large granules of *G. africanum* might also influence the settling velocity of the pollen. Unfortunately, the sporoderm ultrastructure was studied for only one African *Gnetum* species and we do not know whether such large infratectal granules are characteristic for all African species or *G. africanum* only. The African species include *G. africanum* and *G. buchholzianum* Engl. along with the two recently distinguished species *G. interruptum* E.H.Biye and *G. latispicum* E.H.Biye (Biye et al. 2014). To date no experimental studies on the pollination mode of African species of *Gnetum* have been carried out, but macromorphological traits of African species of *Gnetum* are similar to other species, and their habitat (humid rainforests of Cental Africa) indicates that they are entomophilous too (Biye 2013). On the other hand, *Ephedra* species with supposedly different pollination syndromes are characterized by a similar pollen morphology.

Another feature of *Gnetum* pollen most probably associated with the exine structure is its presumably poor preservation, and as a result, a complete absence of *Gnetum* pollen in the fossil record. Two reproductive structures related to *Gnetum* have been described so far: *Siphonospermum simplex* Rydin et Friis (Rydin and Friis 2010) and *Khitania columnispicata* Guo, Sha, Bian et Qui (Guo et al. 2009), both from the Lower Cretaceous of Yixian Formation, China. Of these, Guo et al. (2009) reported that even though pollen grains in situ could not be obtained from male strobili, in pollen assemblages from the same beds pollen grains similar to those of extant *Gnetum* were found. However, no photo of the pollen was shown.

The absence of *Gnetum* pollen in the fossil record is believed to be due to their poor fossilization potential. The latter depends on many factors (Havinga 1967; Hesse et al. 1999). *Gnetum* species grow in humid areas and their pollen grains "spend" a rather short time in the air prior to the germination, so the pollen grains are normally not exposed to, or adapted to, dry conditions. Also, like many species from similar environments, the pollen grains have a thin sporoderm. However, as pollen grains of *Gnetum* are resistant to acetolysis, it is more probable that their small size, thin tectum, and thin ectexine could cause considerable folding and deformation, and the pollen may have been frequently overlooked in fossil assemblages.

An interesting feature of the *Gnetum* sporoderm is that in some sections the "island" part and the inner exine part (endexine and probable foot layer) are rather loosely connected (Fig. 2a–d; see also Gullväg 1966; Meyer-Melikian 1994). Wodehouse (1935) noted that when dried, *Gnetum* pollen grains contract and the exine crumples irregularly. He also thought that there is no permanent harmomegathic mechanism in the pollen. While this kind of sporoderm can fold rather easily in dry conditions, still it has some potential to accommodate changes in volume. The lamellate endexine along with the granular ectexine composed of

islands and valleys allow certain plasticity. Granules seem to be more densely arranged in the central part of the island in *Gnetum* (e.g., Kurmann 1992, Figs. 2f, i, 3b, c, e), which also can facilitate small harmomegathic movements. A similar granule gradient in the infratectum is also often observed in pollen of *Ephedra* and *Welwitschia*. It is possible though that during fossilization of *Gnetum*-like pollen the outer part of the ectexine may easily separate from the rest and thus these sporoderm parts become undefinable. Another simple explanation may be the unique morphology of *Gnetum* pollen, which does not match our generally agreed idea of gymnosperm pollen. In case of a dispersed pollen of this kind one should use SEM and TEM to distinguish *Gnetum* from some similar angiosperm pollen (e.g., *Peperomia* and *Verhuellia* have similar exine ornamentation, Samain et al. 2010). Misinterpretation of some Araceae and ephedroid pollen also occurs and should be carefully checked using electron microscopy (see Hesse et al. 2000; Hesse and Zetter 2007).

Therefore, to distinguish *Gnetum* pollen in the fossil state it is important to study its exine ultrastructure and to compare it with known data on the pollen of modern *Gnetum* species and similar angiosperm taxa. It is also important to study the exine ultrastructure of neotropical and more African species of *Gnetum* to understand the possible influence of geographic isolation in pollen evolution within this genus.

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