

Reproductive morphology in the *Gnetum cuspidatum* group (Gnetales) and its implications for pollination biology in the Gnetales

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Background and aims – The Gnetales include the extant genera *Gnetum*, *Ephedra* and *Welwitschia*. They are usually functionally dioecious, but male cones often have sterile (but pollination drop-producing) ovules in addition to male units. There are, however, exceptions, i.e. most species of *Ephedra* and African species of *Gnetum*. Furthermore, the literature contains conflicting information on the Asian *Gnetum cuspidatum*. One study states that sterile ovules are present in this species; another that they are absent. The latter also claims that male cones secrete nectar instead, which is interesting because nectar has only been suggested to be present in four gymnosperm species. Here we aim to elucidate whether or not sterile ovules are present in male cones of *G. cuspidatum* and related taxa, evaluate evidence for nectar being present in gymnosperms and discuss implications for pollination biology.

Methods – Male cones from relevant taxa were examined using a dissecting microscope and scanning electron microscopy.

Key results – Sterile ovules are present in *G. cuspidatum* and the related *G. macrostachyum*, *G. microcarpum*, *G. diminutum* and *G. loerzingii*, but they are minute, hidden among hairs, and easily overlooked. No indications of nectar or nectaries were found and their presence in Asian species of *Gnetum* is questioned.

Conclusions – Insect pollination is probably ancestral in the Gnetales. Like most species of *Gnetum*, members of the *G. cuspidatum* group have sterile ovules in male cones, and they can thus attract pollinators to both male and female plants using sweet pollination drops. Although it is possible that these species, in addition, produce extraovular reward for pollinators, we find no such evidence. Instead, it seems plausible that pollination drops have been mistaken for (extraovular) nectar. However, African species of *Gnetum* have unisexual male cones. Have they developed another means of pollinator reward in male plants or are they wind-pollinated as are their ephedran analogues?

Key words – Gymnosperms, entomophily, evolution, nectar, pollination drops, taxonomy.

INTRODUCTION

The enigmatic plant order Gnetales continues to engage plant systematists. These peculiar seed plants include the extant genera *Gnetum* L. (c. forty species), *Ephedra* L. (c. sixty species) and the monotypic *Welwitschia* Hook.f. *Gnetum* is distributed in tropical rainforests in South America, Africa and Southeast Asia. *Ephedra* consists of plants that live in arid environments in Asia, Europe, northern Africa, western North America and South America. *Welwitschia mirabilis* Hook.f. is endemic to the Namib Desert.

The Gnetales are clearly monophyletic (Crane 1985, Doyle 1996, Price 1996, Chaw et al. 1997, 2000), but their relationship to other seed plants has long been uncertain. They share some morphological similarities with the angiosperms, such as a form of double fertilization (Friedman 1990, Fried-

man & Carmichael 1996, Carmichael & Friedman 1996) and vascular tissue that resembles that found in the angiosperms (MacDuffie 1921, Muhammad & Sattler 1982). In fact, the Gnetales also have, as have the angiosperms, enclosing structures around the ovules/seeds (i.e. seed envelopes) (figs 1, 2 & 3A–C), but all these structures differ in details from those of angiosperms (see e.g. Thompson 1918, Endress 1996) and most scientists consider the similarities examples of convergent evolution. In support of this, studies based on molecular data place the Gnetales among gymnosperms (Chaw et al. 1997, 2000, Hansen et al. 1999, Bowe et al. 2000, Hajibabaei et al. 2006, Rydin & Korall 2009). There are also morphological similarities between the Gnetales and other gymnosperms, although many are plesiomorphic. One example is the presence of pollination drops, since long recognised as an ancient feature in seed plants (Doyle 1945, Rothwell 1977).

The taxonomy of *Gnetum*, the genus in focus in the present study (fig. 1), is insufficiently determined. The latest monograph of the genus with thorough species descriptions was made a long time ago (Markgraf 1929), and several species have been described since. Markgraf (1929) divided the, at the time, 28 species into two sections, which in turn were divided into two and three subsections respectively. Hou et al. (2015) have, based on molecular data, confirmed some of the sections and subsections and questioned others, and the credibility of species delimitations can often be questioned. A taxonomic revision of *Gnetum* is thus needed.

Gnetum (as well as the entire Gnetales and several other gymnosperms) are dioecious, or rarely monoecious (Hooker 1890, Pearson 1929, Fu et al. 1999). In *Gnetum* the reproductive structures are arranged in lax and elongated spike-like “cones”, in which male and/or female units are positioned together with hair-like extensions at several distinct “levels”, on so-called collars (Strasburger 1872, Lotsy 1899, Thompson 1916, Markgraf 1929, Pearson 1929, Maheshwari & Vasil 1961). The collars have one of two different shapes (fig. 1A–C) and this character forms the basis for Markgraf’s (1929) taxonomic classification: *G.* sect. *Gnemonomorphi* Markgr. (= *G.* sect. *Erecta* Griff.; Griffith 1859), *G.* sect. *Gnetum* (Price 1996), and *G.* sect. *Cylindrostachys* Markgr. (= *G.* sect. *Scandentia* Griff.; Griffith 1859). The former section has low, disk-shaped and distinctly separated collars and the latter has densely placed and cylindrical to funnel-shaped collars (Markgraf 1929). The male cones are morphologically bisexual but functionally unisexual. On each collar, male units are arranged in three to six whorls (Strasburger 1872, Lotsy 1899, Thompson 1916, Markgraf 1929, Pearson 1929, Maheshwari & Vasil 1961, Endress 1996) (fig. 1). The microsporangiophores, usually with two distally positioned microsporangia, are enclosed in fused bract pairs, from which they protrude when mature (fig. 1A). Distally of the male units there is normally a whorl of female units (Strasburger 1872, Lotsy 1899, Thompson 1916, Markgraf 1929, Pearson 1929, Maheshwari & Vasil 1961), each comprising an ovule surrounded by bract-derived seed envelope(s). The female cones, on the other hand, are both functionally and morphologically unisexual and have usually only one whorl of fertile female units on each collar (Strasburger 1872, Lotsy 1899, Thompson 1916, Markgraf 1929, Pearson 1929, Maheshwari & Vasil 1961). For simplicity, we will refer to female units as ‘ovules’ even though gnetalean ovules are enclosed in seed envelopes.

As mentioned above, the Gnetales (as most other gymnosperms) have pollination drops (fig. 1A & B) produced by the nucellus (Strasburger 1872, Pearson 1929, Ziegler 1959, Takaso 1990). The main function of the pollination drops is to receive pollen and transport it through the micropyle to the nucellus (Strasburger 1872, Doyle 1945, Nepi et al. 2009). In the Gnetales, the pollination drop is sweet, which indicates an additional function, namely as reward for pollinators (Karsten 1893, van der Pijl 1953, Ziegler 1959, Kato et al. 1995, Endress 1996). An entomophilous pollination system is common among angiosperms but in gymnosperms it is only present in the Gnetales (Porsch 1910, van der Pijl 1953, Bino et al. 1984a, 1984b, Kato & Inoue 1994, Kato et al. 1995, Wetschnig & Depisch 1999) and in the Cy-

adales (Norstog 1987, Tang 1987, Mound & Terry 2001, Wilson 2002). In *Gnetum*, the pollination biology has only been investigated in three Asian species, *Gnetum gnemon* L., *G. cuspidatum* Blume and *G. luofuense* C.Y. Cheng but the observations indicate pollination by nocturnal insects (van der Pijl 1953, Kato & Inoue 1994, Kato et al. 1995, Corlett 2001). Pollen grains of *Gnetum* are sticky although they lack pollenkitt (Hesse 1980), and they have a spinulose surface (fig. 3E & F) (Gillespie & Nowicke 1994). Furthermore the cones secrete an easily detectable scent, which additionally indicates an entomophilous pollination system (van der Pijl 1953, Kato & Inoue 1994, Kato et al. 1995, Endress 1996).

Species with sterile ovules in the male cones occur in all extant gnetalean genera. This common theme has often been considered analogous rather than homologous because the bisexuality is at different levels of organization in *Welwitschia* compared to *Ephedra* and *Gnetum* (topic reviewed e.g. by Martens 1971, Crane 1985, Endress 1996). However, it has also been argued that the feature nevertheless is homologous and evolved through considerable reduction of reproductive branching systems with proximal male cones and distal female cones (Mundry & Stützel 2004). In male cones of *Welwitschia*, each reproductive unit is morphologically bisexual and has a distal sterile ovule surrounded by six microsporangiophores (Hooker 1863, Pearson 1929). In *Ephedra* there is only one extant species that has sterile ovules in the male cones, namely *Ephedra foeminea* Forssk. (C. Rydin, Stockholm University, Sweden, unpubl. res.; see also Porsch 1910, Endress 1996), the sister of the remaining *Ephedra* species (Rydin & Korall 2009). By contrast, almost all *Gnetum* species (where the male cones are known) have male cones with sterile ovules (Pearson 1912, Markgraf 1929, Markgraf 1972, Fu et al. 1999). As in *Ephedra* but in contrast with *Welwitschia*, reproductive units of *Gnetum* are unisexual, i.e. the ovules with surrounding seed envelopes each constitute separate reproductive units.

Even though the ovules of male plants cannot produce viable seeds (Haycraft & Carmichael 2001) they secrete pollination drops. This means that insects are attracted to both male and female plants of *Gnetum*, despite the fact that the plants are functionally unisexual. There are, however, some exceptions. The African species, *G. africanum* Welw., *G. buchholzianum* Engl., *G. interruptum* E.H. Biye and *G. latispicum* E.H. Biye have morphologically unisexual male cones (Pearson 1912, Markgraf 1929, Biye et al. 2014). Pearson (1912: 614) reports only a single exception to this common theme, seen in a specimen of *G. africanum*. In addition, the literature contains conflicting information on the Asian species *G. cuspidatum*. One study claims that sterile ovules are present in its male cones (Markgraf 1929); another says they are absent (Kato et al. 1995). The reported lack of ovules in male cones of some species is interesting because such species cannot attract pollinators to male plants by the use of pollination drops. Kato et al. (1995) claim that, instead of pollination drops, extraovular nectar is secreted between the microsporangiophores and on the collars of *G. cuspidatum*. They further state that their other study object, *Gnetum gnemon* L. var. *tenerum* Markgr., secretes a very small amount of nectar on the bract pairs that enclose the microsporangiophores. There are, however, no photos or



Figure 1 – Gross morphology of *Gnetum*: A, *Gnetum gnemon*, two male cones with male units (each comprising a microsporangiophore enclosed in a fused bract pair) and female units (each comprising a sterile ovule enclosed in a bract-derived seed envelope) on collars; B, *Gnetum gnemon*, showing pollination drops secreted by the sterile ovules; C, *Gnetum cuspidatum*, parts of a male cone (Korthals s.n., S). Microsporangial bract pairs are visible between collars. On the distal-most collar, hairs are exposed; D, *Gnetum cuspidatum*, field photo of young male cone (Photograph: Johannes Lundberg, Swedish Museum of Natural History).

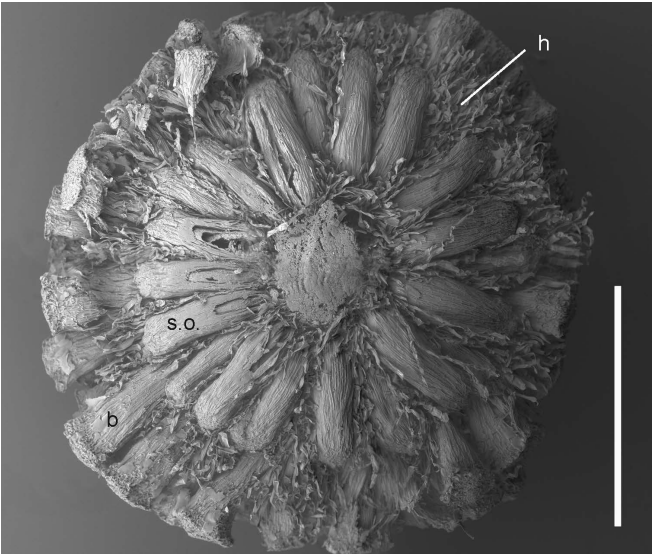


Figure 2 – Transverse section of a male cone of *G. cuspidatum* (Korthals s.n., S). Female units (i.e. sterile ovules enclosed in a bract-derived seed envelope) are arranged in a whorl above the whorls of male units (each comprising a microsporangiophore enclosed in a fused bract pair). To make the ovules visible, most of the hairs were removed but the photo still shows some remaining hairs. Scanning electron micrograph composed of four separate photos. Abbreviations: s.o. = sterile ovule; b = bract of male unit; h = hairs. Scale bar: 2 mm.

other documentation that supports these statements. Corlett (2001) writes that also *G. luofuense* secretes nectar but it is not specified from where it comes. The lack of supportive data and photos in these studies is unfortunate because nectar/nectaries have only been suggested present in four gymnosperms: *G. cuspidatum* (Kato et al. 1995), *G. gnemon* var. *tenerum* (Kato & Inoue 1994, Kato et al. 1995), *G. luofuense* (Corlett 2001) and *Ephedra aphylla* Forssk. (Bino et al. 1984a, 1984b). Otherwise, nectar and nectaries or equivalent structures are present only in angiosperms (Nepi et al. 2009). As in those studies and most studies in the literature, we define nectar as a source of pollinator reward that is *not* a pollination drop. Although the sweet pollination drops of the Gnetales could be functionally considered a kind of ‘nectar’ they are typically not named so because pollination drops are present in almost all gymnosperms, including the wind-pollinated conifers and *Ginkgo*. Pollination drops of most gymnosperms have a low sugar concentration and their function is in most cases not that of pollinator reward (see also Nepi et al. 2009 and above). In passing, it should be mentioned that there are structures in some ferns that are called nectaries (Koptur et al. 1982). These are unlikely to be homologous with nectaries in angiosperms, nor do they have any function for pollination as ferns lack pollen and the reproductive system of seed plants.

Hence, the questions addressed in the present study are: Does *G. cuspidatum* lack sterile ovules in its male cones? If they are absent, what structure secretes the sweet liquid that has been repeatedly observed on their male cones? What is this ‘nectar’ that Kato et al. (1995) speak of and from where is it secreted? These questions are important, partly to find

Table 1 – Study material.
*Even though this specimen could not be determined to *G. diminutum* with certainty, it most probably belongs in clade N of Hou et al. (2015) and is therefore used in the present study. For simplicity, it is referred to as *G. diminutum* in the text.

Voucher	Species	Determinavit
Korthals s.n. (S)	<i>G. cuspidatum</i> Blume	R. Florin
Singh 27411 (L)	<i>G. cuspidatum</i> Blume	F. Markgraf
Meijer 3124 (L)	<i>G. loerzingii</i> Markgr.	F. Markgraf
Meijer 1964a (L)	<i>G. macrostachyum</i> Hook.f.	F. Markgraf
Larsen et al. 41375 (MO)	<i>G. macrostachyum</i> Hook.f.	H. Won
Amin et al. 93873 (L)	<i>G. cf.* diminutum</i> Markgr.	Amin et al.
Kostermans 1372 (L)	<i>G. microcarpum</i> Blume	F. Markgraf

out who is right about the sterile ovules in *G. cuspidatum*, Markgraf (1929) or Kato et al. (1995), but also as a foundation to understand mechanisms and patterns of pollination biology in the Gnetales and other seed plants.

MATERIALS AND METHODS

Since the taxonomy is uncertain and species determination in *Gnetum* can be difficult, we have chosen to examine both *G. cuspidatum* and some closely related taxa (clade N in Hou et al. 2015, and the corresponding “*cuspidatum* clade” in Won & Renner 2006). We selected material determined by specialists of the Gnetales, such as F. Markgraf and R. Florin, whenever possible. We investigated seven herbarium specimens (from L, MO and S; acronyms following Thiers 2015) representing the *G. cuspidatum* group (two of *G. cuspidatum*, one of *G. diminutum* Markgr., two of *G. macrostachyum* Hook.f., one of *G. microcarpum* Blume and one of *G. loerzingii* Markgr.) (table 1). The latter species has never been included in a phylogenetic study but is, according to Markgraf (1929), closely related to the others.

Male cones were dissected and observed using a stereomicroscope (Nikon SMZ800), a light microscope (Zeiss Axio-star) and two scanning electron microscopes (JEOL JSM-7401F and Hitachi TM 3000). The material was softened in hot water and detergent and morphological structures were prepared by using a pair of tweezers and a razor blade. For scanning electron microscopy, dry plant material was mounted on aluminium stubs using sticky tape, and sputter coated with gold particles for 55 s at 10 mA. Micrographs were taken to illustrate the structures.

RESULTS

General description of reproductive morphology in the examined taxa

All specimens had densely placed cylindrical to funnel-shaped collars. In each collar there was normally a single whorl of female units (sterile ovules surrounded by seed envelopes) distal to several whorls of male units (bract-mi-

crosporangiphore complexes) (fig. 2). Surrounding these structures there was a variable number of multicellular, unbranched, uniseriate hairs (figs 1C & 2). Longitudinal sections of the sterile ovules were made to verify that these structures in fact were ovules, especially since *G. cuspidatum* (Singh 27411, L) had some ovules with an outer shape similar to that of the fused bract pairs that enclose the microsporangiphores (compare fig. 3A & D). The sections revealed ovules with a single seed envelope, an integument that is apically extended to form a micropylar tube, and a nucellus (fig. 3A–C).

Species descriptions of male reproductive morphology of examined specimens

***Gnetum cuspidatum*, Korthals s.n. (S)** – The male cones, which were close to pollination stage of development, were at least 3 cm long (incomplete information) and 4–5 mm wide; there were at least 10 collars (incomplete information); hairs were abundantly present and consisted of elongated cells; there were about 20 sterile ovules in a single distal whorl, obliquely ovoid but laterally flattened and with a protracted acuminate apex, 1.3 mm long and 0.6–0.7 mm wide at their widest point.

***Gnetum cuspidatum*, Singh 27411 (L)** – The male cones, which were close to pollination stage of development, were at least 2 cm long (incomplete information) and 4–5 mm wide; there were at least 9 collars (incomplete information); hairs were abundantly present and consisted mostly of rounded cells but elongated cells occurred too; there were about 30 sterile ovules arranged in 2(–3) whorls, obliquely and narrowly obovoid but laterally flattened and with an acute-rounded-truncate apex, 1–1.3 mm long and 0.4–0.5 mm wide at their widest point.

***Gnetum loerzingii*, Meijer 3124 (L)** – The male cone was clearly fertile with protruding microsporangiphores, and was approximately 3 cm long and 6–7 mm wide; there were 11 collars; hairs were abundantly present and consisted of both rounded and elongated cells; there were about 20 sterile ovules in a single distal whorl, obliquely and narrowly ovoid but laterally flattened and with a protracted acuminate apex, 1.3–1.8 mm long and 0.3–0.6 mm wide at their widest point.

***Gnetum macrostachyum*, Meijer 1964a (L)** – The developmental stage of the cones was unclear, but probably juvenile, due to the fact that the collars were tightly spaced and cylindrical, enclosing the reproductive structures completely. The male cones were 7–9 cm long and 7 mm wide; there were 17 collars; hairs were richly present but less than in previous specimens, and consisted of elongated cells; there were about 20 sterile ovules in a single distal whorl, obliquely ovoid but laterally flattened and with a rounded-acute apex, 1–1.3 mm long and 0.4–0.6 mm wide at their widest point.

***Gnetum macrostachyum*, Larsen et al. 41375 (MO)** – The male cones were probably in a juvenile stage due to the fact that the collars were still stacked together. The male cones were 2.2–2.5 cm long and 5–6 mm wide; there were 15–17 collars; hairs were richly present but, as in the former, less than in previous specimens, and consisted of elongated cells; there were about 25 sterile ovules, obliquely and narrowly

ovoid with an acute apex, 0.6 mm long and 0.2 mm wide at their widest point.

***Gnetum diminutum*, Amin et al. 93873 (L)** – The male cones were probably in a juvenile stage due to the fact that the collars were still stacked together. The male cones were 5–12 cm long and 3 mm wide; there were 6–13 collars; hairs were scarcely present and consisted of slightly elongated cells when present; there were about 20 sterile ovules, obliquely obovoid but laterally flattened and with an acute apex, 0.4 mm long and 0.13 mm wide at their widest point.

***Gnetum microcarpum*, Kostermans 1372 (L)** – The male cones were probably juvenile, but closer to a fertile stage than previous specimens. The male cones were 1.4–1.7 cm long and 3–4 mm wide; there were 10–11 collars; hairs were scarcely present and consisted of elongated cells when present; there were about 20 sterile ovules, weakly oblique and broadly ovoid but laterally flattened and with an acute apex, 0.8–0.9 mm long and 0.4–0.5 mm wide at their widest point.

DISCUSSION

Gnetum cuspidatum has sterile ovules in its male cones

This study clearly shows that sterile ovules are present in the male cones of *G. cuspidatum* and related species. The finding is in accordance with Markgraf's (1929) observations but conflicts with those of Kato et al. (1995) who state that sterile ovules are absent in the male cones of *G. cuspidatum*. Kato et al. (1995) studied pollination biology in two *Gnetum* species, *G. gnemon* var. *tenerum* and *G. cuspidatum*, and report that the former has sterile ovules that secrete pollination drops while the latter lacks these and instead secretes nectar "between microsporangiphores and on the collars". It is not clear how they examined the material but morphological descriptions appear to be based on field observations only. This might explain why they did not observe the sterile ovules in *G. cuspidatum* because there is a considerable difference in the appearance of male cones in *G. cuspidatum* and *G. gnemon*. In *G. gnemon* the collars are disc-shaped (low and flattened) with distinct internodes between them (fig. 1A), which makes it easy to distinguish the structures at each collar. By contrast, the reproductive structures of *G. cuspidatum* are partly hidden in the cylindrical and tightly spaced collars (fig. 1C & D). The sterile ovules of *G. gnemon* are also larger than those of *G. cuspidatum*, and are clearly visible above the male units. Yet another complicating factor is the presence of hairs that cover the ovules. *Gnetum cuspidatum* has much more hairs than has *G. gnemon*, and its sterile ovules are concealed by hairs and very difficult to detect in the field. Without dissection under a stereo microscope, it is easy to get the impression that the sweet pollination drops that are secreted from the hidden ovules instead represent nectar of an unknown source that emerges on the collars and male units, which is what Kato et al. (1995) report.

Is nectar present in gymnosperms?

Kato et al. (1995) write that they saw nectar on the male cones of *G. cuspidatum*, and that also *G. gnemon* var. *tenerum* secrete small amounts of nectar from the bracts

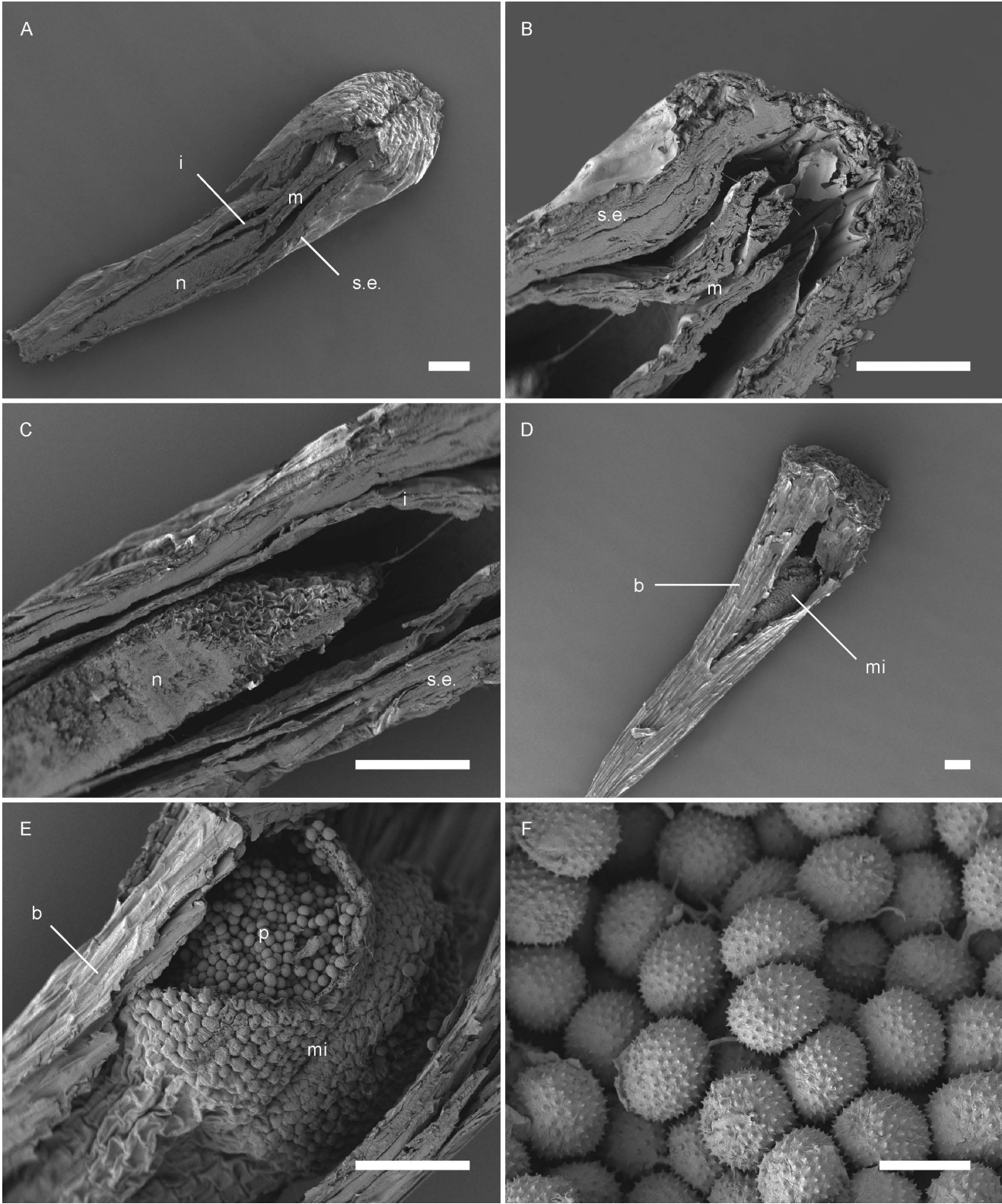


Figure 3 – Reproductive structures in *G. cuspidatum*, Singh 27411 (L) (A–C) and *Korthals s.n. (S)* (D–F): A, longitudinal section of a female unit: a sterile ovule enclosed in a bract-derived seed envelope; B, longitudinal section of the micropylar end of a female unit; C, longitudinal section of an ovule’s central part, showing nucellus, integument and the single enclosing seed envelope; D, male unit with microsporangia visible through a torn opening in the bract; E, close-up of D showing microsporangia with pollen; F, pollen. Scanning electron micrographs. Abbreviations: i = integument; n = nucellus; m = micropyle; s.e. = seed envelope; b = bract of male unit; mi = microsporangia; p = pollen. Scale bars: A–E = 100 μ m; F = 10 μ m.

of the male units. There are, however, no photos or other documentation to support the statements, nor is there any information in the literature that indicates the presence of a secreting structure in bracts and collars. It has been suggested that the hairs could have a secreting function (Kubitzki 1990), and they could therefore be the source of the small amount of fluid that Kato et al. (1995) observed in *G. cuspidatum* and *G. gnemon* var. *tenerum*. We have, however, not observed any fluid coming from the male units of *G. gnemon* in our greenhouse. Instead, pollination drops often drip from the ovules and land on the bracts and collars beneath (fig. 1A & B). Regarding *G. cuspidatum*, it is difficult to be certain since we do not have access to the plant in the field or in cultivation, but nectar secretion by hairs seems to be an unsatisfactory explanation. The quantity and appearance of hairs differed considerably among the examined individuals, and in two specimens, hairs were almost absent. Considering our observations of *G. gnemon*, it is probable that the reported liquid on the collars and male units of *G. cuspidatum* represents pollination drops that have leaked from the sterile ovules that secrete these drops, as we frequently observe in *G. gnemon*.

A final possible explanation for the (probably incorrect) conclusions in Kato et al. (1995) is that there seems to be a printing error in the reference they used when they determined the specimen to *G. cuspidatum*. In the flora they used, Flora Malesiana volume four (Markgraf 1950), the male cones of *G. cuspidatum* are described as follows: “♂ Inflorescences cauline, simple, thick, about 6 cm long, 5 mm thick, pendulous. ♂ Flowers numerous, 80–100, obconic, 2 mm high; sporophyll exerted by 1 mm; sterile flowers 10, acute, ovate.” This differs from Markgraf’s monograph from 1929 in that a ♀-symbol (represented by the word *feminini* in Markgraf 1929) is missing between the words ‘sterile’ and ‘flowers’ in Markgraf (1950). It should thus be: sterile ♀ flowers 10. Because the ♀-symbol was missing, readers may easily get the impression that the statement refers to (sterile)

male flowers and that ovules are not mentioned, thus missing, in male cones of *G. cuspidatum*. This has also led to the incorrect assumption that extraovular nectar must exist in order for the species to attract pollinators to its male plants and that the observed liquid represents such nectar. This assumption has unfortunately received a lot of attention; *Gnetum* is often cited as almost the only example where nectar is present in gymnosperms (with reference to Kato et al. 1995).

It is of course possible that nectar is present as an additional attractant, and/or that intraspecific variation occurs, but there is currently no documentation or structural evidence of that. Kato (2005) argues that another species, *Gnetum leptostachyum* Blume, produces both pollination drops from ovules and nectar from bracts, referring to Kato et al. (1995). *Gnetum leptostachyum* is, however, not mentioned in Kato et al. (1995), and it is difficult to assess if the statement is a mistake or if Kato et al. (1995) made this observation even though it is not reported in the paper.

Thus, although nectar in the form of leaking phloem sap (no nectaries formed) has been reported for some angiosperms (Vogel 1997), the simplest solution is that nectar is missing in *Gnetum* and that the liquid observed on collars and microsporangiophore bracts of the male units of some species of *Gnetum* is pollination drops, secreted from sterile ovules, that have leaked onto other parts of the cones. The lack of convincing evidence for nectar secretion, in combination with the results of the present study, which show that Asian species reported to lack sterile ovules in fact have such structures in the male cones, make the presence of extraovular nectar in *Gnetum* improbable, in particular since no other gymnosperms (with the possible exception of *Ephedra aphylla*) produce nectar. The uncritical use of the term nectar has unfortunately repeatedly been taken as evidence that the Gnetales have nectaries/nectar (see examples in Endress 1996, Hufford 1996, Corlett 2001, Haycraft & Carmichael 2001, Nepi et al. 2009).

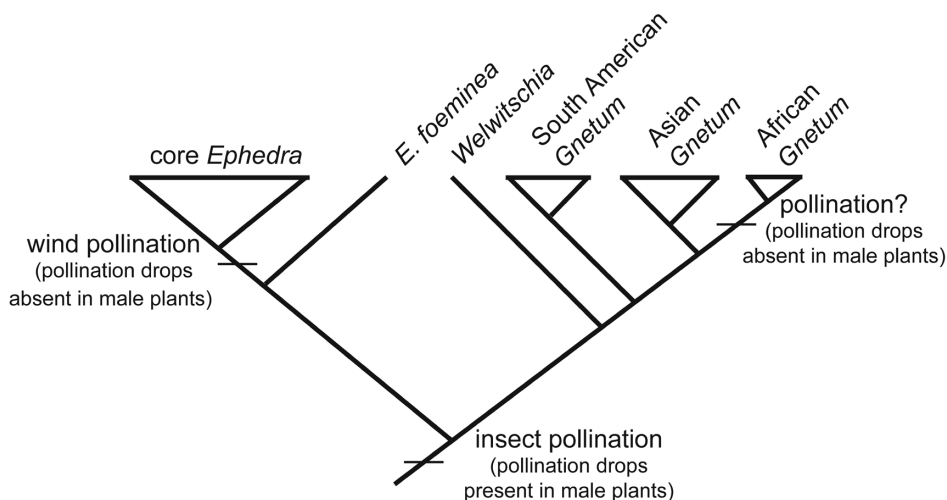


Figure 4 – Pollination biology in the Gnetales, its evolution, and link to presence/absence of pollination drop producing ovules in male cones (i.e. male plants). The hypothesis is based on results in the present study and in Markgraf (1929), Rydin & Korall (2009), Bolinder et al. (2014) and Hou et al. (2015). Plants that lack ovules cannot use pollination drops as attraction and reward for pollinators. In *Ephedra*, species that lack sterile ovules in male plants are (with the potential exception of *E. aphylla*) considered wind-pollinated. The pollination mode of African species of *Gnetum*, which also lack sterile ovules in male plants, has to our knowledge never been studied.

Comparisons with literature data and implications for species delimitations and pollination biology

Species delimitations in the clade we have studied are probably more diffuse than hitherto known. Hou et al. (2015) have already begun to unravel some of the taxonomy, but additional studies using substantially increased sampling of taxa from all regions and proposed species/subspecies/varieties, studied using molecular and morphological data, are needed and will require extensive field work in remote tropical areas. In Markgraf's monograph (1929) the species descriptions are detailed but it is not clear how many specimens were used for the species descriptions, nor if they are based on dried or fresh material. Markgraf (1929) describes the number of sterile ovules as well as their shape, length and width in some species, but for other species, information is scarce or absent.

Something that has become clear during the work with the present study is that our observations of shape and number of ovules differ from what is stated in the literature. All male cones examined here have about twenty sterile ovules or more in each collar, which is considerably more than what is specified in the respective descriptions of *G. cuspidatum*, *G. diminutum* and *G. macrostachyum*. For example, Markgraf's (1929) description of *G. cuspidatum* states that there are about ten sterile ovules in each collar, but specimen Singh 27411 (L) (determined to *G. cuspidatum* by Markgraf) is here shown to have closer to thirty sterile ovules in each collar. It is possible that Markgraf made his assessments mainly based on outer and vegetative morphology, and may therefore have overlooked the true number of sterile ovules, in particular in members of *G. sect. Cyndrostachys* where these structures are difficult to detect. The same problem exists with all species we have examined except *G. microcarpum*, for which the number of ovules detected in the present study agrees with Markgraf's (1929) report. Of the approximately forty species where information about the number of sterile ovules is available, only four species are described to have twenty sterile ovules per collar or more. Conceivably, most species should be re-examined for the character.

Our observation that sterile ovules of male cones of *Gnetum* have only one seed envelope (in contrast with two seed envelopes found in fertile ovules of female cones of the genus), is in accordance with earlier observations (Strasburger 1872, Pearson 1915, 1929). However, shape and size of the sterile ovules differ repeatedly from the descriptions in the literature, although it must be taken into account that some of the material observed here was juvenile and therefore may differ in size compared to Markgraf's reports (1929). Regarding ovule shape, our observations show that the ovules are bent apically, in an outward-downward direction, in almost all observed specimens; only one specimen had straight ovules, the young male cone in *G. macrostachyum* (Larsen et al. 41375, MO). This differs markedly from information in the literature, where *G. macrostachyum* (and *G. diminutum*) are described as having apically curved ovules (Markgraf 1929), while the sterile ovules of *G. cuspidatum*, *G. microcarpum* and *G. loerzingii* are described as erect and non-curved. Further, according to Markgraf (1929), dimorphism regarding ovule shape appears to exist in some species (e.g.

G. microcarpum and *G. macrostachyum*), which may explain the presence of curved ovules in one specimen of *G. macrostachyum* and straight ovules in the other.

A possible reason for the curved shape of ovules of some species is that organ shape is partly determined by pressure from other organs during development (Endress 2008). In this case it appears as if the shape of the collars influences that of the ovules. For species of *G. sect. Cyndrostachys*, with very short internodes, it should also be of adaptive value to have bent ovules that are exposed between collars. In those species, an upright ovule would secrete its pollination drops onto the above positioned collar. If the ovules instead are bent, the pollination drops are efficiently exposed to pollinators between microsporangioophores and the drops probably contribute to making the pollen grains stickier. These could be factors that have a positive effect on the pollination system in *G. sect. Cyndrostachys* and could possibly explain the higher diversity in this clade compared to the remaining genus. Similar kinds of secondary pollen presentation are also suggested for other insect pollinated members of the Gnetales, i.e. *Welwitschia* (Hufford 1996) and *E. foeminea*, in which large amounts of pollen are trapped in the sweet pollination drops produced by sterile ovules in the male cones (C. Rydin, Stockholm University, Sweden, pers. obs.).

CONCLUSIONS

In combination with information in the literature, the results of the present study indicate that all species of *Gnetum* except the African species have sterile ovules in their male cones. Bisexual cones occur in all three genera of the Gnetales and may be an ancestral trait (Thompson 1916, Lloyd & Wells 1992, Mundry & Stützel 2004). The feature is probably linked to insect pollination, which indicates that insect pollination too is an ancestral condition, at least in the extant Gnetales (fig. 4). *Welwitschia*, which lives in one of Earth's driest places, the Namib Desert, is insect-pollinated (Wetschnig & Depisch 1999), and has thus preserved the ancestral state despite the fact that wind pollination appears beneficial in the environment the plant inhabits. The sister species of the remaining *Ephedra*, *E. foeminea*, has also retained insect pollination, and has sterile ovules and pollination drop production in male cones (Bolinder et al. 2014, Rydin & Bolinder 2015). Most other *Ephedra* species are considered wind-pollinated (Bolinder et al. 2014) and these same species lack sterile ovules and pollination drops in their male cones.

An interesting question is whether the loss of sterile ovules in the African species of *Gnetum* indicates a shift to wind pollination also within *Gnetum*. The African species, like the remaining species of the genus, inhabit tropical areas where insect pollination should be beneficial. But if the male plants lack sterile ovules and thus also pollination drops, how can they entice pollinators to male cones? The African species do not only occur in humid forests but in a wide range of habitats including more dry and open areas (Biye et al. 2014), where wind pollination could function. Or have they retained insect pollination and developed another means of pollinator reward in male plants?

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REFERENCES

- Bino R.J., Dafni A., Meeuse A.D.J. (1984a) Entomophily in the dioecious gymnosperm *Ephedra aphylla* Forssk. (=E. alte C.A.Mey.), with some notes on *Ephedra campylopoda* C.A.Mey. 1. Aspects of the entomophilous syndrome. Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen, Series C-Biological and Medical Sciences 87: 1–13.
- Bino R.J., Devente N., Meeuse A.D.J. (1984b) Entomophily in the dioecious gymnosperm *Ephedra aphylla* Forssk. (=E. alte C.A.Mey.), with some notes on *Ephedra campylopoda* C.A.Mey. 2. Pollination droplets, nectaries, and nectarial secretion in *Ephedra*. Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen, Series C -Biological and Medical Sciences 87: 15–24.
- Biye E.H., Balkwill K., Cron V.C. (2014) A clarification of *Gnetum* L. (Gnetaceae) in Africa and the description of two new species. *Plant Systematics and Evolution* 300: 263–272. <http://dx.doi.org/10.1007/s00606-013-0879-6>
- Bolinder K., Humphreys A.M., Ehrlén J., Alexandersson R., Ickert-Bond S.M., Rydin C. (2014) Pollination mechanisms in the ancient gymnosperm clade *Ephedra* (Gnetales). In: *Pollination in Ephedra* (Gnetales). Licentiate thesis, Stockholm University, Stockholm, Sweden.
- Bowe L.M., Coat G., dePamphilis C.W. (2000) Phylogeny of seed plants based on all three genomic compartments: extant gymnosperms are monophyletic and Gnetales' closest relatives are conifers. *Proceedings of the National Academy of Sciences of the United States of America* 97: 4092–4097. <http://dx.doi.org/10.1073/pnas.97.8.4092>
- Carmichael J.S., Friedman W.E. (1996) Double fertilization in *Gnetum gnemon* (Gnetaceae): its bearing on the evolution of sexual reproduction within the Gnetales and the anthophyte clade. *American Journal of Botany* 83: 767–780. <http://dx.doi.org/10.2307/2445854>
- Chaw S.M., Zharkikh A., Sung H.M., Lau T.C., Li W.H. (1997) Molecular phylogeny of extant gymnosperms and seed plant evolution: analysis of nuclear 18S rRNA sequences. *Molecular Biology and Evolution* 14: 56–68. <http://dx.doi.org/10.1093/oxfordjournals.molbev.a025702>
- Chaw S.M., Parkinson C.L., Cheng Y.C., Vincent T.M., Palmer J.D. (2000) Seed plant phylogeny inferred from all three plant genomes: monophyly of extant gymnosperms and origin of Gnetales from conifers. *Proceedings of the National Academy of Sciences of the United States of America* 97: 4086–4091. <http://dx.doi.org/10.1073/pnas.97.8.4086>
- Corlett R.T. (2001) Pollination in a degraded tropical landscape: a Hong Kong case study. *Journal of Tropical Ecology* 17: 155–161. <http://dx.doi.org/10.1017/S0266467401001109>
- Crane P.R. (1985) Phylogenetic analysis of seed plants and the origin of angiosperms. *Annals of the Missouri Botanical Garden* 72: 716–793. <http://dx.doi.org/10.2307/2399221>
- Doyle J. (1945) Developmental lines in pollination mechanisms in the Coniferales. *Scientific Proceedings of the Royal Dublin Society* 24: 43–62.
- Doyle J.A. (1996) Seed plant phylogeny and the relationships of Gnetales. *International Journal of Plant Sciences* 157: S3–S39. <http://dx.doi.org/10.1086/297401>
- Endress P.K. (1996) Structure and function of female and bisexual organ complexes in Gnetales. *International Journal of Plant Sciences* 157: S113–S125. <http://dx.doi.org/10.1086/297407>
- Endress P.K. (2008) The whole and the parts: relationships between floral architecture and floral organ shape, and their repercussions on the interpretation of fragmentary floral fossils. *Annals of the Missouri Botanical Garden* 95: 101–120. <http://dx.doi.org/10.3417/2006190>
- Friedman W.E. (1990) Double fertilization in *Ephedra*, a nonflowering seed plant: its bearing on the origin of angiosperms. *Science* 247: 951–954. <http://dx.doi.org/10.1126/science.247.4945.951>
- Friedman W.E., Carmichael J.S. (1996) Double fertilization in Gnetales: implications for understanding reproductive diversification among seed plants. *International Journal of Plant Sciences* 157: S77–S94. <http://dx.doi.org/10.1086/297405>
- Fu L.-K., Yu Y.-F., Gilbert M.G. (1999) Gnetaceae. In: Wu Z.-Y., Raven P.H. (eds) *Flora of China*, vol. 4: 102–105. Beijing, Science Press; St Louis, Missouri Botanical Garden Press.
- Gillespie L.J., Nowicke J.W. (1994) Systematic implications of pollen morphology in *Gnetum*. *Acta Botanica Gallica* 141: 131–139. <http://dx.doi.org/10.1080/12538078.1994.10515146>
- Griffith W. (1859) XXIII. Remarks on *Gnetum*. *Transactions of the Linnean Society of London* 22: 299–312. <http://dx.doi.org/10.1111/j.1096-3642.1856.tb00101.x>
- Hajibabaei M., Xia J.N., Drouin G. (2006) Seed plant phylogeny: gnetophytes are derived conifers and a sister group to Pinaceae. *Molecular Phylogenetics and Evolution* 40: 208–217. <http://dx.doi.org/10.1016/j.ympev.2006.03.006>
- Hansen A., Hansmann S., Samigullin T., Antonov A., Martin W. (1999) *Gnetum* and the angiosperms: molecular evidence that their shared morphological characters are convergent, rather than homologous. *Molecular Biology and Evolution* 16: 1006–1009. <http://dx.doi.org/10.1093/oxfordjournals.molbev.a026176>
- Haycraft C.J., Carmichael J.S. (2001) Development of sterile ovules on bisexual cones of *Gnetum gnemon* (Gnetaceae). *American Journal of Botany* 88: 1326–1330. <http://dx.doi.org/10.2307/3558344>
- Hesse M. (1980) Pollenkitt is lacking in *Gnetum gnemon* (Gnetaceae). *Plant Systematics and Evolution* 136: 41–46. <http://dx.doi.org/10.1007/BF00985310>
- Hooker J.D. (1863) I. On *Welwitschia*, a new genus of Gnetaceae. *Transactions of the Linnean Society of London* 24: 1–48. <http://dx.doi.org/10.1111/j.1096-3642.1863.tb00151.x>
- Hooker J.D. (1890) Gnetaceae. *The flora of British India* 5: 640–643.
- Hou C., Humphreys A.M., Thureborn O., Rydin C. (2015) New insights into the evolutionary history of *Gnetum* (Gnetales). *Taxon* 64: 239–253. <http://dx.doi.org/10.12705/642.12>
- Hufford L. (1996) The morphology and evolution of male reproductive structures of Gnetales. *International Journal of Plant Sciences* 157: S95–S112. <http://dx.doi.org/10.1086/297406>
- Karsten G. (1893) Zur Entwicklungsgeschichte der Gattung *Gnetum*. *Beiträge zur Biologie der Pflanzen* 6: 337–382.
- Kato M., Inoue T. (1994) Origin of insect pollination. *Nature* 368: 195. <http://dx.doi.org/10.1038/368195a0>

- Kato M., Inoue T., Nagamitsu T. (1995) Pollination biology of *Gnetum* (Gnetaceae) in a lowland mixed dipterocarp forest in Sarawak. *American Journal of Botany* 82: 862–868. <http://dx.doi.org/10.2307/2445972>
- Kato M. (2005) Ecology of traplining bees and understory pollinators. In: Roubik D.W., Sakai S., Hamid Karim A.A. (eds) *Pollination ecology and the rain forest: Sarawak studies. Ecological Studies* 174: 128–133. New York, Springer. http://dx.doi.org/10.1007/0-387-27161-9_11
- Koptur S., Smith A.R., Baker I. (1982) Nectaries in some neotropical species of *Polypodium* (Polypodiaceae): preliminary observations and analyses. *Biotropica* 14: 108–113. <http://dx.doi.org/10.2307/2387739>
- Kubitzki K. (1990) Gnetatae. In: Kramer K.U., Green P.S. (eds) *The families and genera of vascular plants*, vol. 1: 378–391. Berlin-Heidelberg, Springer.
- Lloyd D.G., Wells M.S. (1992) Reproductive biology of a primitive angiosperm, *Pseudowintera colorata* (Winteraceae), and the evolution of pollination systems in the Anthophyta. *Plant Systematics and Evolution* 181: 77–95. <http://dx.doi.org/10.1007/BF00937588>
- Lotsy J. (1899) Contributions to the life history of the genus *Gnetum*. *Annales du Jardin Botanique de Buitenzorg* 16: 46–114.
- MacDuffie R.C. (1921) Vessels of the gnetalean type in angiosperms. *Botanical Gazette* 71: 438–445. <http://dx.doi.org/10.1086/332865>
- Maheshwari P., Vasil V. (1961) *Gnetum*: 32–42. New Delhi, Council of Scientific & Industrial Research.
- Markgraf F. (1929) Monographie der Gattung *Gnetum*. *Bulletin du Jardin Botanique de Buitenzorg* 10: 407–511.
- Markgraf F. (1950) Gnetaceae. *Flora Malesiana* 4: 336–347.
- Markgraf F. (1972) Gnetaceae. *Flora Malesiana* 6: 944–949.
- Martens P. (1971) Les Gnétophytes. In: Zimmermann W., Carlquist S., Ozenda P., Wulff H.D. (eds) *Handbuch der Pflanzenanatomie, Spezieller Teil*, vol. 12. Berlin, Borntraeger.
- Mound L.A., Terry I. (2001) Thrips pollination of the central Australian cycad, *Macrozamia macdonnellii* (Cycadales). *International Journal of Plant Sciences* 162: 147–154. <http://dx.doi.org/10.1086/317899>
- Muhammad A.F., Sattler R. (1982) Vessel structure of *Gnetum* and the origin of angiosperms. *American Journal of Botany* 69: 1004–1021. <http://dx.doi.org/10.2307/2442898>
- Mundry M., Stützel T. (2004) Morphogenesis of the reproductive shoots of *Welwitschia mirabilis* and *Ephedra distachya* (Gnetales), and its evolutionary implications. *Organisms Diversity & Evolution* 4: 91–108. <http://dx.doi.org/10.1016/j.ode.2004.01.002>
- Nepi M., von Aderkas P., Wagner R., Mugnaini S., Coulter A., Pacini E. (2009) Nectar and pollination drops: how different are they? *Annals of Botany* 104: 205–219. <http://dx.doi.org/10.1093/aob/mcp124>
- Norstog K. (1987) Cycads and the origin of insect pollination. *American Scientist* 75: 270–279.
- Pearson H.H.W. (1912) On the microsporangium and microspore of *Gnetum*, with some notes on the structure of the inflorescence. *Annals of Botany* 26: 603–620.
- Pearson H.H.W. (1915) IX. Notes on the morphology of certain structures concerned in reproduction in the genus *Gnetum*. *Transactions of the Linnean Society of London, Series 2 Botany* 8: 311–332. <http://dx.doi.org/10.1111/j.1095-8339.1915.tb00287.x>
- Pearson H.H.W. (1929) Gnetales: 52–99, 106, 121–122, 133. Cambridge, Cambridge University Press.
- Porsch O. (1910) *Ephedra campylopoda* C.A.Mey., eine entomophile Gymnosperme. *Berichte der Deutschen Botanischen Gesellschaft* 28: 404–412.
- Price R.A. (1996) Systematics of the Gnetales: a review of morphological and molecular evidence. *International Journal of Plant Sciences* 157: S40–S49. <http://dx.doi.org/10.1086/297402>
- Rothwell G.W. (1977) Evidence for a pollination-drop mechanism in Paleozoic pteridosperms. *Science* 198: 1251–1252. <http://dx.doi.org/10.1126/science.198.4323.1251>
- Rydin C., Korall P. (2009) Evolutionary relationships in *Ephedra* (Gnetales), with implications for seed plant phylogeny. *International Journal of Plant Sciences* 170: 1031–1043. <http://dx.doi.org/10.1086/605116>
- Rydin C., Bolinder K. (2015) Moonlight pollination in the gymnosperm *Ephedra* (Gnetales). *Biology Letters* 11: 20140993. <http://dx.doi.org/10.1098/rsbl.2014.0993>
- Strasburger E. (1872) Die Coniferen und die Gnetaceen: eine morphologische Studie: 155–158, 236–238, 265–273. Jena, Hermann Dabiz.
- Takaso T. (1990) Pollination drop time at the Arnold Arboretum. *Arnoldia* 50: 2–7.
- Tang W. (1987) Insect pollination in the cycad *Zamia pumila* (Zamiaceae). *American Journal of Botany* 74: 90–99. <http://dx.doi.org/10.2307/2444334>
- Thiers B. (2015) Index Herbariorum: A global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. Available at <http://sweetgum.nybg.org/ih/> [accessed 7 Aug. 2015].
- Thompson W.P. (1916) The morphology and affinities of *Gnetum*. *American Journal of Botany* 3: 135–184. <http://dx.doi.org/10.2307/2435095>
- Thompson W.P. (1918) Independent evolution of vessels in Gnetales and angiosperms. *Botanical Gazette* 65: 83–90. <http://dx.doi.org/10.1086/332191>
- van der Pijl L. (1953) On the flower biology of some plants from Java with general remarks on fly-traps (species of *Annona*, *Artocarpus*, *Typhonium*, *Gnetum*, *Arisaema* and *Abroma*). *Annales Bogorienses* 1: 77–99.
- Vogel S. (1997) Remarkable nectaries: structure, ecology, organophyletic perspectives. 1. Substitutive nectaries. *Flora* 192: 305–333.
- Wetschnig W., Depisch B. (1999) Pollination biology of *Welwitschia mirabilis* Hook. f. (Welwitschiaceae, Gnetopsida). *Phyton-Annales Rei Botanicae* 39: 167–183.
- Wilson G.W. (2002) Insect pollination in the cycad genus *Bownia* Hook. ex Hook. f. (Stangeriaceae). *Biotropica* 34: 438–441. <http://dx.doi.org/10.1111/j.1744-7429.2002.tb00557.x>
- Won H., Renner S.S. (2006) Dating dispersal and radiation in the gymnosperm *Gnetum* (Gnetales) – Clock calibration when out-group relationships are uncertain. *Systematic Biology* 55: 610–622. <http://dx.doi.org/10.1080/10635150600812619>
- Ziegler H. (1959) Über die Zusammensetzung des "Bestäubungstropfens" und den Mechanismus seiner Sekretion. *Planta* 52: 587–599. <http://dx.doi.org/10.1007/BF01914757>

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