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**Phylogenetic and taxonomic studies in *Macaranga*,  
*Mallotus* and other acalyphoid genera (Euphorbiaceae s.s.)**

Kulju, K.K.M.

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**RE-DISCOVERY AND PHYLOGENETIC POSITION OF THE  
 INCERTAE SEDIS GENUS *AFROTREWIA* (EUPHORBIACEAE):  
 MORPHOLOGICAL, POLLEN AND MOLECULAR EVIDENCE**

KRISTO K.M. KULJU<sup>1</sup>, RAYMOND W.J.M. VAN DER HAM<sup>1</sup> & FRANS J. BRETELER<sup>2</sup>  
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SUMMARY

The monotypic tropical African genus *Afrotrewia* (with *A. kamerunica*; Euphorbiaceae s.s.), previously unclassified because of the lack of extant specimens, is re-described and neotypified based on newly collected material. In addition to Cameroon, its distribution is extended to Gabon. The phylogenetic position of *Afrotrewia* and that of the putatively related *Necepsia* was determined by using two chloroplast markers (*trnL-F* and *rbcL*), which were sequenced for these genera and analysed together with a previously published Euphorbiaceae s.s. dataset. In addition, the pollen of *Afrotrewia* was studied. The morphology of the specimens studied matches well with the original description. The phylogenetic analyses show that *Afrotrewia* is part of the core acalyphoid clade and not related to *Necepsia*, but its precise position is still ambiguous. The pollen of *Afrotrewia* resembles the pollen of several other acalyphoid genera; these are, however, scattered throughout the phylogenetic tree, indicating homoplasy in pollen structure. As no taxa potentially congeneric with *Afrotrewia* could be identified, we conclude that the generic status for this taxon is justified.

**Key words:** *Afrotrewia*, Cameroon, Euphorbiaceae, Gabon, molecular phylogeny, palynology.

INTRODUCTION

In the latest classification of the family Euphorbiaceae (334 genera; Radcliffe-Smith, 2001), *Afrotrewia* Pax & K. Hoffm. was one of the three genera placed *incertae sedis* due to the lack or scarcity of material. This monotypic genus was first collected in Cameroon by Mildbraed in the early 20<sup>th</sup> century. Pax & Hoffmann (1914) described it as part of the series *Bernardiiformes*, and assumed a close relationship to the genus *Necepsia* Prain. Later, *Afrotrewia* was mentioned again by Pax & Hoffmann (1931) and by Govaerts et al. (2000), but overlooked in the classification of Webster (1994b). The type collection of *Afrotrewia kamerunica* Pax & K. Hoffm. was lost with the destruction of the Berlin herbarium (B), and no isotypes have been found, and, until recently, no additional material has apparently been collected or identified as *A. kamerunica*. Lately, however, the senior author (F. Breteler) discovered two specimens, collected recently near the type locality in southern Cameroon, which match the original description. Also, two older collection from the same region were recovered, and one unidentified Euphorbiaceae from Gabon proved to represent this species as well.

<sup>1</sup>Nationaal Herbarium Nederland, Universiteit Leiden Branch, P.O. Box 9514, 2300 RA Leiden, The Netherlands.

<sup>2</sup>Nationaal Herbarium Nederland, Herbarium Vadense, Gen. Foulkesweg 37, 6703 BL Wageningen, The Netherlands.

In this paper we neotypify *Afrotrewia*, provide an amended description and confirm its distinctiveness. Furthermore, the purpose of the study was to determine the phylogenetic position of the genus and to test the hypothesis that *Afrotrewia* is closely related to *Necepsia* (Acalyphoideae tribe Bernardieae in Radcliffe-Smith, 2001). For this purpose we sequenced two chloroplast markers, *trnL-F* and *rbcL*, for these genera, and analysed them together with the large uniovulate Euphorbiaceae (Euphorbiaceae s.s.) dataset of Wurdack et al. (2005). A description of the pollen of *Afrotrewia*, based on light microscopy (LM) and scanning electron microscopy (SEM), is also provided.

#### MATERIALS & METHODS

For the LM and SEM observations of *Afrotrewia kamerunica* pollen, the collection from Cameroon, South Prov., *Tchouto et al. 3260* (WAG) was studied with the methods described in Van De Ven & Van Der Ham (2006).

The DNA samples from the following WAG herbarium specimens were extracted for this study (in parantheses the newly obtained Genbank accession numbers for *trnL-F* and *rbcL* sequences, respectively): *Afrotrewia kamerunica* Pax & K. Hoffm., *Tchouto et al. 3260* (DQ991516, DQ991513); *Necepsia afzelii* Prain, Gabon, Ogooué-Lolo, *Breteler & Breteler 12307* (DQ991514, DQ991511); *Necepsia afzelii* Prain, Gabon, Ogooué-Lolo, *Breteler et al. 15189* (DQ991515, DQ991512).

The general DNA laboratory methods, the amplification of *trnL-F*, sequencing and sequence assembling follow Kulju et al. (2007, Chapter 2). The *rbcL* gene was amplified in two overlapping parts with the primer pairs 1F+724R and 636F+1460R (Fay et al., 1997) under the same PCR conditions as *trnL-F*. The newly obtained sequences were manually added to the previously published uniovulate Euphorbiaceae datamatrix of Wurdack et al. (2005) using MacClade v4.08 (Maddison & Maddison, 2001). In the phylogenetic analyses the *trnL-F* character exclusions of Wurdack et al. (2005; the ambiguously aligned regions) were followed, and gaps treated as missing data.

The parsimony analyses were conducted with PAUP\* v4.0b10 (Swofford, 2003). To speed up the analyses of the large dataset, the parsimony ratchet (Nixon, 1999) was used to search for the most parsimonious trees. The ratchet batch files for PAUP\* were generated with PRAP v.1.21 (Müller, 2004), conducting ten rounds of Random Addition Sequence (RAS) and TBR each followed with 200 ratchet iterations (25% of characters given double weight). Clade support was assessed by bootstrap analysis (Felsenstein, 1985), with 2000 pseudoreplicates and saving a single tree obtained with one RAS+TBR per pseudoreplicate.

The models of molecular evolution to be used in Bayesian inference of phylogeny were selected using Akaike Information Criterion (AIC) as implemented in MrModeltest v.2.2 (Nylander, 2004). The GTR+G+I model was chosen for both *trnL-F* and *rbcL*. The Bayesian analyses were done with MrBayes v.3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003), using the default priors of the program. Two simultaneous runs were done for each analyses, having 3 heated and 1 cold chain with default temperature (0.2). Markov chains were sampled every 100<sup>th</sup> generation. Analyses were run until average standard deviation of split frequencies approached 0.01, indicating that two runs converged onto a stationary distribution. Additionally, the plot of generation vs. log probability was inspected after the run to ensure that sta-

tionarity was reached, and to determine the burn-in. Depending on the dataset, 1 200 000–4 000 000 generations were run, and c. 10–30% of the samples were discarded as burn-in.

## RESULTS

**Pollen morphology of *Afrotrewia kamerunica***—Pollen grains medium-sized ( $P \times E = 21.2 \times 21.0 \mu\text{m}$ ), more or less obtusely triangular to circular in polar view, elliptic to circular in equatorial view ( $P/E = 1.01$ ). *Apertural system* 3-colporate. *Colpi* narrow,  $14\text{--}15 \times c. 2 \mu\text{m}$ , with up to  $2 \mu\text{m}$  wide, psilate margos, without operculum, without costae; colpus membrane irregularly covered with scabrae. *Endoapertures* lalongate,  $2.5\text{--}3.5 \times 7\text{--}9 \mu\text{m}$ , with indistinct lateral sides and slightly thickened polar sides (costae endocolpi). *Exine*  $1.3\text{--}2 \mu\text{m}$  thick, distinctly stratified (LM), with a thin nexine, a thin columellate infratectum (as thick as nexine) and a thick tectum (as thick as nexine and infratectum together). *Ornamentation* microreticulate, without echinae. Perforations up to  $0.7 \mu\text{m}$  in diameter; smallest along the margos (Fig. 7.1).

**Molecular phylogenetic study**—The initial analyses of separate *trnL-F* and *rbcL* datasets with a full sampling of Euphorbiaceae s.s. taxa placed *Afrotrewia* and *Necepsia* unambiguously in the Acalyphoideae s.s. clade (results not shown). In the subsequent analyses, only this clade was included and the genera from the “acalyphoid grade” (*Pogonophora* Miers ex Benth., *Clutia* L., *Chaetocarpus* Thwaites, *Pera* Mutis and *Neoscortechinia* Hook.f. ex Pax) were used as outgroups (see Fig. 3 in Wurdack et al., 2005). Parsimony and Bayesian analyses of combined *trnL-F* and *rbcL* data resulted in highly similar trees (Fig. 7.2), and in both analyses *Afrotrewia* is a member of the well-supported core acalyphoid clade, and specifically, forms a clade with the subclades A2 and A3 of Wurdack et al. (2005). Parsimony places *Afrotrewia* sister to A2+A3, whereas in Bayesian results these three elements occur in a polytomy. The analyses of only the *rbcL* data positioned *Afrotrewia* sister to subclade A3, while the *trnL-F*

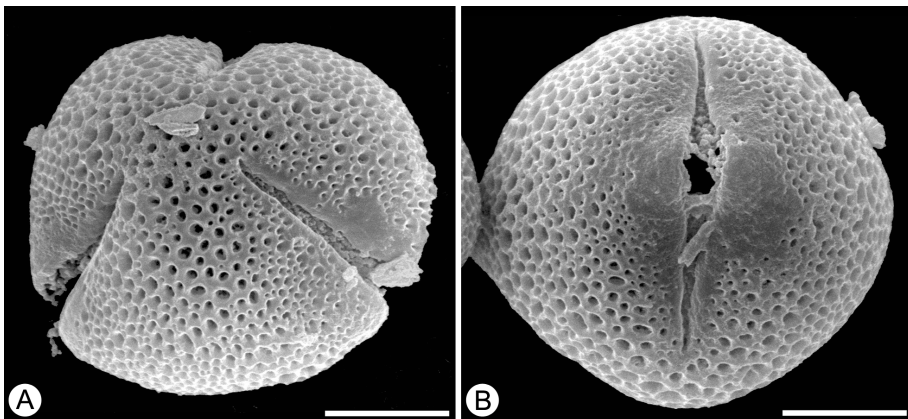


Fig. 7.1. Pollen of *Afrotrewia kamerunica* (SEM). A. Polar view. B. Equatorial view. Scale bar =  $5 \mu\text{m}$ .

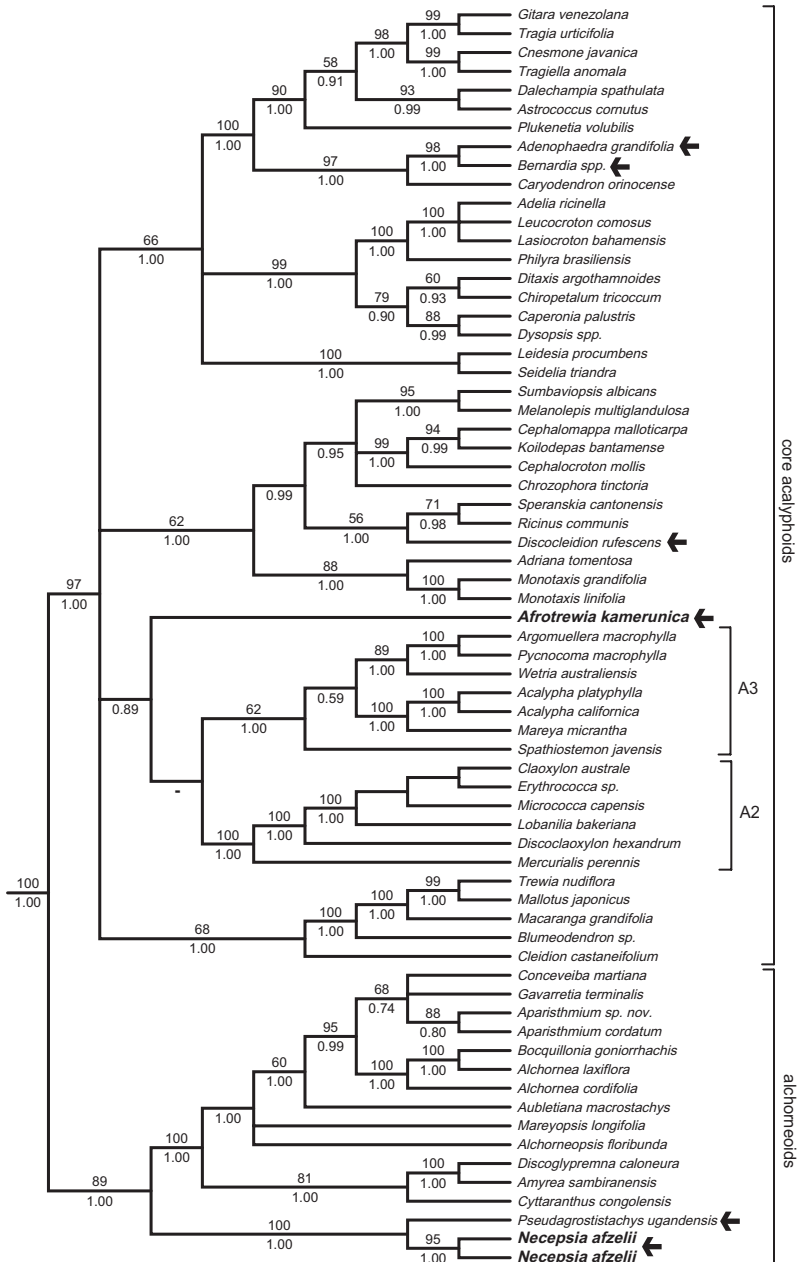


Fig. 7.2. Strict consensus of the parsimony analysis (tree length 1923, CI 0.536, RI 0.672) of the combined *trnL-F* and *rbcL* data in Acalyphoideae s.s. (from the uniovulate Euphorbiaceae dataset of Wurdack et al., 2005) with newly sequenced *Afrotrewia* and *Necepsia* (in bold). Outgroups omitted from the tree. Parsimony bootstrap values shown above the branches, Bayesian posterior probabilities below. -: node does not exist in the Bayesian majority consensus. Taxa with pollen resembling *Afrotrewia* marked with arrows. Clade names follow Wurdack et al. (2005)

analysis placed it in the large basal polytomy of the core acalyphoid clade (results not shown). In all analyses, the two *Necepsia* samples were placed in the alchorneoid clade, grouping with *Pseudagrostistachys* Pax & K. Hoffm. and/or *Paranecepsia* Radcl.-Sm. (the latter sampled only for *rbcL*). The general topology and support of our combined analysis agrees to a large extent with the published analysis of the complete dataset (Wurdack et al., 2005) with the exception of a few clades being less resolved (unsupported nodes only).

## TAXONOMIC TREATMENT

### AFROTREWIA

*Afrotrewia* Pax & K. Hoffm. in Engl., Pflanzenr. IV.147.vii: 14. 1914. — Type: *Afrotrewia kamerunica* Pax & K. Hoffm.

Shrubs to small trees; presumably dioecious. *Indumentum* consisting of stellate (to simple) minute hairs, whitish to pale rusty. *Stipules* triangular, caducous to somewhat persistent, margin entire, apex acute. *Leaves* alternate, simple; petiole short, terete to somewhat angular, apically pulvinate; blade (ovate to) elliptic to obovate, symmetric, papery, margin subentire to shallowly serrate, extrafloral nectaries present on both surfaces; venation pinnate. *Inflorescences* unisexual; *bracts* triangular, persistent, apex acute; *bracteoles* at the base of inflorescence branches and pedicels, resembling the bracts but smaller; staminate inflorescences slender, (axillary to) terminal panicles with 1 or 2 orders of branching, flowers 1–3 per node; pistillate ones terminal, 2- or 3-flowered racemes, flowers single per node. *Flowers* actinomorphic, pedicellate; corolla absent. *Staminate flowers*: calyx 2- or 3-lobed, valvate; receptacle ± conical; disc interstaminal, consisting of several short, hairy glands; stamens 30–40; filaments free, somewhat flattened; anthers basifixed, thecae 2, pendulous, unequally bilobed (inner lobe smaller), latrorse or introrse, longitudinally dehiscent, connective horizontally enlarged, partly covering the thecae, papillose; pistillode absent. *Pistillate flowers*: sepals 5 or 6, imbricate? (buds not seen), triangular; disc annular, thick; ovary 3-locular, 1 ovule per locule, style short, stigmas 3, twice bifid to somewhat irregularly divided, with long papillae adaxially. *Fruits* lobed capsules, opening septicidally, septifragally and loculicidally, column not seen. *Seeds* subglobose, ± smooth, brown and slightly mottled when dry; hilum ± triangular to heart-shaped; testa not fleshy; caruncle absent; mature embryo and endosperm not seen, cotyledons broad, flat.

Distribution — One species in Cameroon and Gabon.

#### 1. *Afrotrewia kamerunica* Pax & K. Hoffm. — Fig. 7.3

*Afrotrewia kamerunica* Pax & K. Hoffm. in Engl., Pflanzenr. IV.147.vii: 14. 1914. — Type: “Südkamerun, Kribi, Randgebirge zwischen Ebolowa und Kampo”, *Mildbraed* 6203 (B, †). Neotype (designated here): Cameroon, South Prov., Campo Ma’an area, Massif des Mamelles, toward the summit, 2°35′32″ N, 9°57′50″ E, alt. 220 m, 18 August 2002, *Tchouto et al.* 3407 (WAG!; isoneotypes: SCA, YA).

Shrub to tree, up to 10 m high; branches terete, young ones somewhat longitudinally ribbed when dry, sparsely (to densely) hairy, glabrescent; flowering branches 0.75–1.5

mm thick. *Bark* pale grey. *Indumentum*: hairs 0.1–0.2(–0.4) mm long. *Stipules* 1–3 × 0.5–1.5 mm, sparsely to densely hairy outside, sparsely hairy inside. *Leaves*: petiole 4–9 mm long, sparsely to densely hairy; blade 10.5–20.5 × 2.7–5.8 cm, length/width ratio 2.7–4.4, base obtuse and minutely emarginate, marginal teeth 9–18 per side, apex acuminate to caudate; upper surface glabrous, basal extrafloral nectaries 1–6, (orbicular to) elliptic, 0.3–0.9 × 0.2–0.6 mm, 1.5–6 mm from petiole insertion, 1–2 mm from midrib, additional extrafloral nectaries 8–20, similar to basal ones but smaller, irregularly dispersed over the blade; lower surface glabrous (to subglabrous on midrib and nerves), extrafloral nectaries 11–20, elliptic, 0.2–0.4 × 0.2–0.3 mm, irregularly dispersed over the blade; venation ± flat above, raised beneath, lateral nerves 9–13 per side, looped and closed near the margin, veins irregularly scalariform to somewhat reticulate, veinlets reticulate. *Staminate inflorescences* up to 12 cm long, rachis basally c. 0.5–0.75 mm thick, sparsely hairy; *bracts* 0.5–2 × 0.25–1 mm (on main axis of panicle bigger than on side-branches), sparsely hairy outside, glabrous inside. *Staminate flowers*: yellow to cream when fresh; pedicels up to 5 mm long, basally hairy, with abscission zone in basal part; buds ovoid, apiculate, glabrous, up to 1.5 mm in diameter; open flowers 2.5–3 mm in diameter; calyx lobes ovate, 1–2 × 1–1.5 mm; filaments 0.5–0.8 mm long, glabrous; anthers c. 0.4 × 0.4 mm. *Pistillate inflorescences* up to 6 cm long, rachis basally 0.5–1 mm thick, sparsely hairy; *bracts* 1–2 × 0.5–0.75 mm, sparsely hairy outside, (sub)glabrous inside. *Pistillate flowers*: pedicel 8–10 mm long, sparsely hairy at apex, densely hairy at base, with basal abscission zone; sepals c. 1 × 0.5 mm, sparsely hairy outside, glabrous inside; disc as high as sepals or somewhat higher, densely hairy; style c. 0.5 mm long; stigmas 1–2 mm long. *Fruits*: reddish green when fresh; cocculus valves c. 10 mm long (intact mature fruits not seen), surface smooth, sparsely to densely hairy, cocculus wall woody, 0.25–0.75 mm thick. *Seeds* 6–7 × 6–6.5 × 6–7 mm, hilum 2.5–3 × 2.5–3 mm.

Distribution and ecology — Cameroon (South Province) and Gabon (Moyen-Ogooué). In riverine and Atlantic biao forests.

Additional specimens studied — Cameroon, South Prov., 27 km from Kribi, Ebo-lowa road, Kienke river, 2°52' N, 10°07' E, 27 January 1970, *Bos 6179* (K!, MA!, WAG!). Cameroon, South Prov., Campo Ma'an area, Massif des Mamelles, 2°33'57" N, 9°56'58" E, alt. 280 m, 23 April 2001, *Tchouto et al. 3260* (KRI, SCA, WAG!). Cameroon, South Prov., Campo Ma'an area, forest near Boussebeliga creek bridge. 2°43' N, 9°52' E, alt. 10 m, 26 October 2001, *Van Andel et al. 4213* (WAG!). Gabon, Moyen-Ogooué, Missanga, c. 0°05' S, 10°45' E, 12 November 1991, *Breteler et al. 10415* (LBV, WAG!).

## DISCUSSION

The newly found material (see above) matches the original description of *Afrotrewia* (Pax & Hoffmann, 1914) well, including the measurements. The only notable discrepancies are the number of stamens (30–40 instead of “± 25”) and the presence of extra-floral nectaries on both upper and lower leaf surfaces instead of only on the upper surface as stated in the original description. Therefore, we are confident that the new material represents the *Afrotrewia* of Pax & K. Hoffm.

Our combined phylogenetic analysis (Fig. 7.2) shows that *Afrotrewia* belongs to

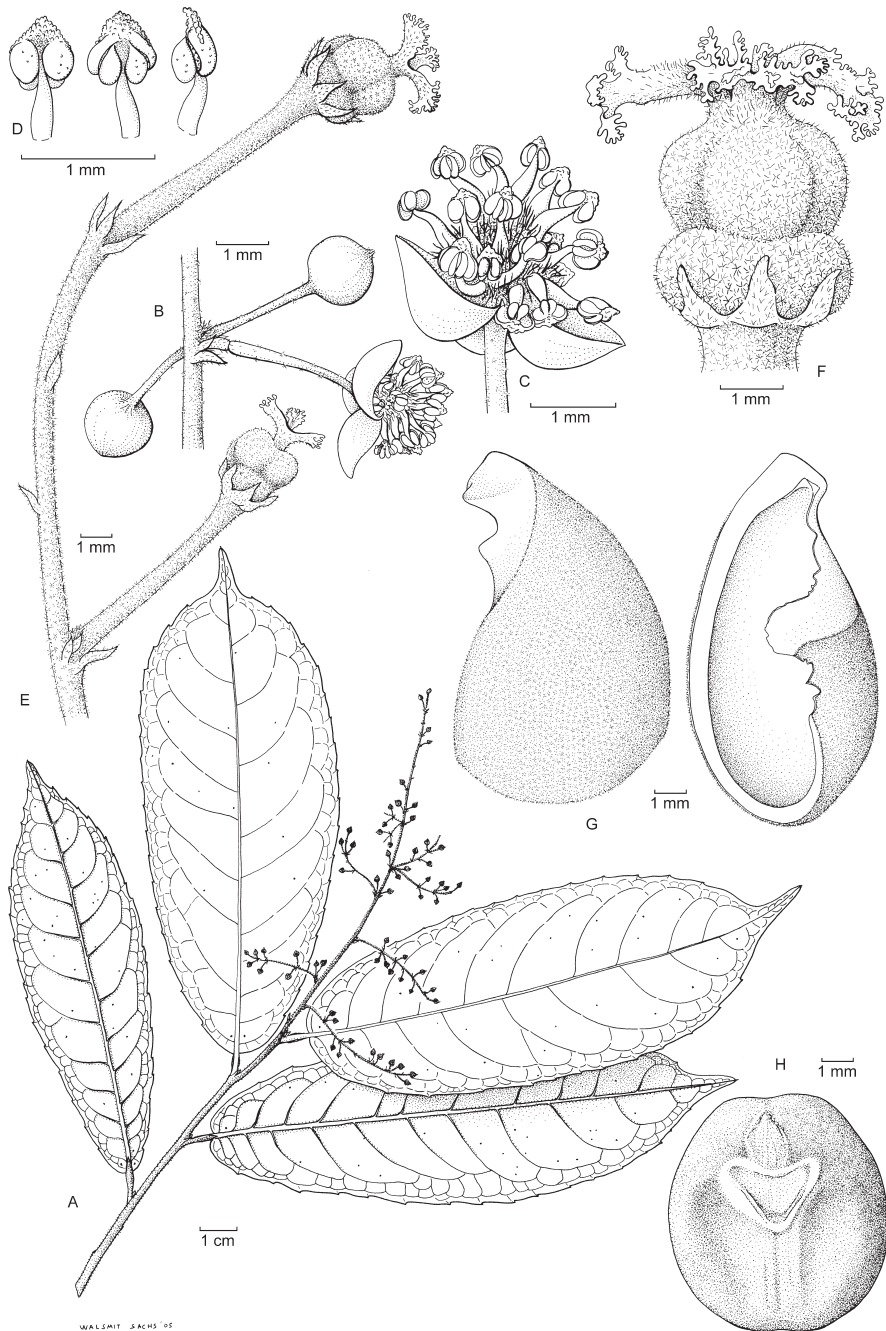


Fig. 7.3. *Afrotrewia kamerunica*. A, habit of staminate branchlet; B, detail of staminate inflorescence; C, staminate flower; D, stamens, dorsal, ventral and lateral view; E, detail of pistillate inflorescence; F, pistillate flower; G, remnants of dehiscent fruit; H, seed. (A, E–H, *Peguy 3407*; B–D, *Bos 6179*).



Euphorbiaceae s.s. subfamily Acalyphoideae s.s. and is placed with strong support in the core acalyphoid clade. It is also obvious that *Afrotrewia* is not closely related to *Necepsia*, as originally suggested<sup>3</sup> (Pax & Hoffmann, 1914), because the latter falls into the separate, well-supported alchorneoid clade. Morphologically, the separation of these two genera is supported by a number of differences, e.g., in indumentum (hairs in *Necepsia* simple instead of stellate) and inflorescence structure (in *Necepsia* axillary, often bisexual, and very short when staminate; in *Afrotrewia* mostly terminal, unisexual, and slender and relatively long when staminate).

*Afrotrewia* is placed together with the core acalyphoid clades A2 and A3 in the combined analyses, and as sister to clade A3 in *rbcL* analyses, but these positions are not supported by the bootstrap analyses or posterior probabilities. Clades A2 and A3 are morphologically diverse, and no apparent morphological synapomorphies shared by them and *Afrotrewia* are currently known. A study with additional genetic markers and a denser taxon sampling might clarify the phylogeny in the core acalyphoids in general and the exact placement of *Afrotrewia* in particular. However, *Afrotrewia kamerunica* has a unique combination of characters within the subfamily Acalyphoideae (indumentum stellate, extrafloral nectaries on both leaf surfaces, inflorescences unisexual and mostly terminal, petals absent, disc interstaminal [staminate] or annular [pistillate]), and none of the unsampled acalyphoid genera are particularly similar to it morphologically. There is thus no reason to question the placement of this species in a genus of its own.

Pollen structure has strongly influenced the classification of Euphorbiaceae, and has been considered to provide important characters from generic to subfamily level (Webster, 1994a). As comprehensive palynological work on the subfamily Acalyphoideae (Nowicke et al., 1998, 1999; Takahashi et al., 2000; Nowicke & Takahashi, 2002) was completed recently, the pollen characters of *Afrotrewia* were expected to inform on its phylogenetic position. Although the medium-sized, spheroidal, 3-colporate pollen of *Afrotrewia kamerunica* represents a rather basic type within the subfamily, the character combination columellate infratectum,  $\pm$  microreticulate ornamentation and colpi with psilate margos, suggests similarities with *Pseudagrostistachys* (tribe Agrostistachydeae) and *Adenophaedra* (Müll. Arg.) Müll. Arg., *Bernardia* Houst. ex Mill., *Discocleidion* (Müll. Arg.) Pax. & K. Hoffm., *Necepsia* and *Paranecepsia* (tribe Bernardieae) (Nowicke & al., 1999). However, in the molecular phylogenetic tree (Fig. 7.2), none of these genera are close to *Afrotrewia*. They are instead scattered throughout the tree, in three different clades, suggesting high levels of homoplasy in these pollen exine characters. Generally, as the molecular phylogenetic results (Wurdack et al., 2005) agree only partly with the current classification strongly influenced by pollen data (Webster, 1994b; Radcliffe-Smith, 2001), it is clear that the importance of the pollen structure in the classification of the subfamily Acalyphoideae needs to be carefully re-evaluated.

<sup>3</sup>Pax & Hoffmann (1914; further mentioned by Radcliffe-Smith, 2001) also indirectly suggested a close relationship of *Afrotrewia* and *Crotonogynopsis* Pax, because both of them are mentioned to have affinities with *Necepsia*. In our results (not shown; *Crotonogynopsis* included only in the *rbcL* dataset) *Crotonogynopsis* is sister to *Acalypha*+*Mareya* in clade A3, and therefore not particularly closely related to *Afrotrewia*.

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